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6. AUTHOR(S)
Albert S. Baldwin, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of North Carolina at Chapel Hill
Chapel Hill, NC 27599-7030

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Human breast cancer is characterized by the altered expression of growth factors, growth factor receptors and kinases. Downstream effectors of these molecules are transcription factors which presumably control aberrant gene expression regulating cell growth. We are studying the potential role of the transcription factor NF-κB in human breast cancer. Our rationale is based on the observations that NF-κB is activated in several human breast cancer cell lines and that certain repetitive DNA elements known as VNTRs, which bind NF-κB, are associated with breast cancer. Our data indicate that NF-κB is required for oncogenic transformation and that this transcription factor protects tumor cells against oncogene-induced and cancer therapy-induced apoptosis. Analysis of human breast cancer tumors indicate that NF-κB subunits p50, p52 and c-Rel are up-regulated in the great majority of tumors as compared to normal adjacent tissue. We are currently exploring whether these NF-κB forms bind to the VNTR elements and contribute to altered gene expression controlled by these elements. Functional experiments are underway to determine if altered NF-κB activity is involved in the initiation and progression of human breast cancer.

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PRINCIPAL INVESTIGATOR: Albert S. Baldwin, Ph.D.

CONTRACTING ORGANIZATION: University of North Carolina
Chapel Hill
Chapel Hill, NC 27599-7030

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Albert S Baldwin 7/28/97

PI - Signature Date

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INTRODUCTION:

Human breast cancer is characterized by altered expression of growth factors, growth factor receptors and kinases (Lippman and Dickson, 1989). Downstream modulators of growth factors and kinases are transcription factors which likely modulate altered responses in breast cancer. Genetic analysis also indicates that other factors are involved in development or progression of human cancer, including repetitive DNA elements called VNTR elements (Krontiris et al, 1993), the recently cloned BRCA1 gene (Futreal et al, 1994), and the tumor suppressor gene p53 (Rotter et al, 1993). Additional emphasis has been placed on the role of estrogen in the development of breast cancer.

NF-κB/Rel proteins are widely distributed and are typically found in the cytoplasm where they are associated with an inhibitor protein called IκB (Beg and Baldwin, 1993; Baldwin, 1996). Upon exposure to inflammatory cytokines or growth factors, NF-κB factors dissociate from the IκB inhibitory proteins and translocate into the nucleus (Finco and Baldwin, 1995). In the nucleus, these dimeric factors regulate transcription of genes that contain the κB binding site. Typically these genes encode proteins involved in immune and inflammation responses but more recent work has shown that genes encoding growth regulatory proteins (such as c-*myc*) are regulated by NF-κB (see Baldwin, 1996). There are presently five members of the NF-κB/Rel family of proteins: p50/NF-κB1, p52/NF-κB2, c-Rel, RelB, and p65/RelA. There are four members of the IκB family: IκBα, IκBβ, IκBε, and Bcl-3. The first 3 forms of IκB function as inhibitors while Bcl-3 appears to function as a transcriptional co-activator with the p50 and p52 subunits of NF-κB (see Results section).

NF-κB and IκB proteins are associated with oncogenesis. For example, members of the NF-κB family of proteins are related to the product of the c-Rel proto-oncogene which is found overexpressed in certain tumor cell lines. Additionally, the p100/NF-κB2 member of the NF-κB family is found translocated in certain lymphomas as is Bcl-3, a member of the IκB family (see Beg and Baldwin, 1993; Baldwin, 1996). Based on our preliminary data, we have proposed that dysregulation of normal NF-κB regulation (i.e., chronic nuclear localization of some forms of NF-κB) may play an important role in the development or progression of human breast cancer. However, new data indicates a second mechanism whereby NF-κB may be activated and separate new data indicates a mechanism whereby NF-κB appears to function in oncogenesis (see Progress/Results section). These new results take on broadly significant implications regarding oncogenesis in general and particularly in regards to breast cancer since it was published last year that oncogenic HER2/Neu (an oncogene activated in a significant number of breast cancers) can activate transcription through NF-κB binding sites (Galang et al., 1996). In fact, our recent data indicate that NF-κB is required for oncogenic transformation and tumorigenesis (see progress/results section).

We have previously found that a breast cancer cell line exhibited constitutive activation of NF-κB. Furthermore, we had found that estrogen treatment of a fibroblast caused enhanced expression of a reporter gene that is controlled by multiple NF-κB sites. Furthermore, some genes shown to be overexpressed in human breast cancer (for example, vimentin and ICAM-1) are known to be regulated by NF-κB. Thus one broad aim of the proposal (Dr. Baldwin's part of the project) was to study the expression of NF-κB/Rel proteins in human breast cancer and to study the functional outcome of this potential overexpression. Our new findings do not alter the aims that were originally planned but they do alter how we approach them (see below). Additionally, these results raise questions regarding whether oncogenes associated with breast cancer (HER2/Neu or TC21) may functionally target NF-κB as part of the transformation process.

A second focus of this proposal is the functional study of the Ha-ras variable number tandem repeat (VNTR) in breast cancer. VNTRs arise from the head to tail concatenation of short sequence motifs. The VNTR at the Ha-ras locus is about 1 kilobase downstream from the Ha-ras 1 proto-oncogene and consists of 30-120 copies of a 28 bp sequence. Krontiris et al (1987) first showed that rare alleles of Ha-ras appear in the genome of cancer patients at a higher frequency

that in non-affected individuals. More recent data indicate that the breast cancer risk due to a rare allele may be greater in African-American women (Garrett et al, 1993).

It has been published that the Ha-ras VNTR appears to bind NF-κB transcription factors, although supershift studies were never performed to verify this protein binding (Trepicchio and Krontiris, 1992). In addition, a number of studies have reported transcriptional enhancement activity of this VNTR (Green and Krontiris, 1993). The broad aims of this part of the proposal (Dr. Conway) are to explore a role for VNTR elements in human breast cancer, evaluate the association of rare alleles with breast cancer and further characterize the factors that bind to the Ha-ras VNTR element.

As stated above, rare alleles of the Ha-ras VNTR occur more frequently in individuals with cancer, including breast cancer, than in those without cancer (Garrett et al., 1993; Krontiris, 1994). Although the mechanism of this is association is unknown, one explanation may be that Ha-ras alleles possess a biological function and the rare alleles may function differently than the common alleles. If the Ha-ras VNTR possesses transcriptional regulatory activity, this function might be affected by variations in allelic sequence. We have found that the various Ha-ras alleles vary not only in repeat copy number but also in the interspersion pattern of repeat sequences along the VNTR. Sequence analysis of individual 28 bp repeats shows that a given repeat may possess either a G or a C at positions 7 or 15, giving rise to four possible repeat types. We have developed a minisatellite variant repeat (MVR)-PCR approach to detect these sequence variants. Using this method, we have shown that VNTR allele length is very tightly linked to MVR internal sequence (Conway et al., 1996). That is, nearly all alleles having the same length also have the same MVR allelotype. In contrast to the common alleles that are detected repeatedly in a population, rare alleles possess unique and disordered sequences (Conway et al., 1996; Conway et al, in preparation). Most rare VNTR alleles begin at the 5' end as one common allele then abruptly switch to either a second recognizable motif or become completely unrecognizable. This suggests that rare MVR alleles arise via recombination involving segments of one or more of the common alleles.

The G/C polymorphisms within the 28 bp repeat subunit could potentially affect transcription factor binding, perhaps by influencing methylation patterns or DNA secondary structure. If we are able to more clearly define the differences between rare and common alleles based upon a combination of transcription factor binding, transcriptional enhancement activity, and structural characteristics such as VNTR length and internal sequence variations, we can identify true rare alleles which predispose to breast cancer. To this end, we proposed to investigate the potential regulatory role of the Ha-ras VNTR by characterizing its interaction with members of the NF-κB/Rel family and other transcription factors. This was to be accomplished by evaluating protein binding to 28 bp VNTR subunits carrying specific G/C polymorphisms, and to longer tandem arrays of subunits generated by MVR from either common or rare alleles. We were also interested in characterizing the binding proteins present in a series of nuclear extracts from cell lines and from breast tumor tissues as well as normal mammary epithelium. Finally, we wanted to determine the biological outcome of VNTR/protein interactions by characterizing transcriptional regulatory activity of common versus rare VNTR sequences. Listed below are the original aims of the grant application:

Aim 1 is to investigate the potential biological function of the Ha-ras VNTR through characterization the nuclear factors that bind to this element with a definite focus on the potential interaction of NF-κB/Rel proteins. Further approaches include studies aimed at addressing potential transcriptional activation properties of the VNTR.

Aim 2 is designed to determine if NF-κB/Rel binding to VNTR elements may be used as a more refined method of identifying patients at risk for breast cancer.

Aim 3 is to analyze relative nuclear and cytoplasmic levels of NF-κB/Rel proteins in normal breast epithelium and in human breast cancer. We will correlate NF-κB expression with activation of certain kinases thought to regulate NF-κB expression and with the status of transcriptional activators shown to regulate NF-κB gene expression.

Aim 4 is to correlate expression of NF-κB with expression of known or suspected prognostic markers for human breast cancer (ICAM-1, urokinase and vimentin). We will determine if the ligand for HER2/Neu, NDF, can induce the expression of NF-κB.

Aim 5 is to determine whether estrogen can regulate gene expression through a κB site and whether this is due to the activation of NF-κB/Rel binding activity.

BODY (Baldwin Laboratory):

Progress/Results (some of these results were described in the last report but papers have now been published/submitted and are now described in more detail):

NF-κB functional activity is controlled by Ras oncogenic alleles. We have initiated our studies using the oncogene Ras and its downstream effector Raf. We show (see Finco et al., attached) that oncogenic Ras or oncogenic Raf potently activates gene expression through an NF-κB binding site. However, oncogenic Ras or Raf do not increase nuclear translocation of NF-κB. This presented an enigma to us: how is it that NF-κB functional activity can be increased without an increase in nuclear NF-κB? So we tested whether the innate transcriptional activity of NF-κB p65 is increased under these conditions. Our experiments indicate that the transcriptional activation domain of the p65 subunit of NF-κB is functionally targeted by oncogenic Ras or oncogenic Raf. Thus, these data indicate that two oncogenes (Ras or Raf) can potently stimulate transcription of NF-κB dependent transcription without inducing nuclear translocation of NF-κB. This is accomplished apparently by the targeting of the transcriptional function of the p65 subunit which exists at low, constitutive levels in most cells.

NF-κB is required for oncogenic Ras to neoplastically transform cells. With the above data in mind, we have asked whether NF-κB is required for oncogenic Ras to transform cells. We now show that the ability of Ras to transform cells is blocked by expression of the NF-κB inhibitor IκBα (see Finco et al., in the appendix). Additionally, we show that cells that have the NF-κB p65 genes deleted are incapable of activation of κB-dependent transcription in response to oncogenic Ras or oncogenic Raf. Thus, these data indicate that the NF-κB subunit p65 is required for oncogenic Ras to transform cells. We are obviously very interested in determining if transformation by the breast cancer oncogene HER2/Neu requires NF-κB (see goals for the upcoming year).

NF-κB functional activity blocks apoptosis. Protection from apoptosis is a critical component in neoplastic transformation and in protection from radiological and chemotherapies (Fisher, 1994). Our data now indicate that NF-κB activation protects cells from killing by several cancer therapies. Data shown in the attached manuscript (Wang et al) indicates that NF-κB protects cells against killing by tumor necrosis factor. The experimental approach was to express a super-repressor form of IκBα (mutated in serines 32 and 36, which results in a protein that can bind to NF-κB but which cannot be degraded) in the HT1080 fibrosarcoma cell line. This cell line was chosen since it is highly resistant to killing by TNF. Expression of IκBα blocks NF-κB nuclear translocation in these cells and correspondingly sensitizes to killing by TNF. Further evidence (not shown) that NF-κB is protective against killing by TNF are the following observations: (1) re-expression of NF-κB subunits in the cells expressing IκBα restores protection against killing, (2) fibroblasts that are null for the p65 NF-κB subunit (from the p65 knockout) show enhanced killing by TNF, and (3) cells that are highly sensitive to killing by TNF do not activate NF-κB in response to this cytokine. Thus, these data strongly indicate that NF-κB protects cells against killing by TNF.

The requirement of NF-κB for Ras-mediated transformation is based on the anti-apoptotic function of NF-κB. The ability of IκBα expression to block Ras-mediated cellular transformation (described above) appears to be based on the ability of NF-κB to block apoptosis. As described in Mayo et al (see attached manuscript), inhibition of NF-κB in the setting of expression of oncogenic Ras leads to apoptosis. This result has important implications for our data described below, suggesting that NF-κB activation in breast cancer may serve as an anti-apoptotic function to prevent oncogene-induced apoptosis.

NF-κB is activated by cancer chemotherapies and radiation and significantly blunts the apoptotic response to these therapies. Since several cancer chemotherapies kill transformed cells

by the induction of apoptosis (Fisher, 1994), we have determined if NF-κB protects against killing by these treatments. We have initially focused on 2 standard cancer therapies: ionizing radiation and daunorubicin. Our data (see Wang et al., 1996, manuscript attached) indicate that the inhibition of NF-κB potently enhances cell killing in response to radiation and daunorubicin treatment. Interestingly, radiation and daunorubicin activate NF-κB but adriamycin does not. Thus, our data indicate that cancer therapies that kill and also activate NF-κB are (at least partially) less effective at inducing apoptosis unless NF-κB activity is blocked. Recent data indicate that camptothecin/CPT-11 activates NF-κB and the inhibition of NF-κB significantly improves the tumor cell killing response (see draft in the appendix). Thus, we propose that inhibitors of NF-κB will potently enhance cell killing by ionizing radiation and daunorubicin (and potentially other therapies).

Analysis of NF-κB expression in human breast cancer: evidence for increased nuclear localization of p50 and p52 NF-κB subunits and Bcl-3. We have largely accomplished one major aspect of Aim 3; that is, analyzing the expression of NF-κB/Rel proteins by immunohistochemical studies of human breast cancer sections. Utilizing the antibody against the human NF-κB p65 subunit, it was found that approximately 30-35% of human breast tumors express extraordinarily high levels of NF-κB, as compared to normal breast epithelium (reported in the last report). Interestingly, the majority of these exhibit cytoplasmic levels of p65, although NF-κB p65/RelA is nuclear in several examples.

Our recent data on nuclear extracts of breast tumors shows that there is a significant increase in NF-κB binding activity in the tumors versus the adjacent tissue controls (see Figure 1 in the Appendix). This data is tabulated in Table 1 below. We noted that the mobility of this form(s) of NF-κB was faster than authentic NF-κB p50/p65. Antibody supershift data indicate that p50 and p52 were included in the gel mobility shift complex (see Figure 2 in the appendix). Additionally, immunoblotting of nuclear extracts versus adjacent tissue controls showed that NF-κB p50, p52 and c-Rel were all expressed in the nucleus of breast cancer cells relative to the adjacent tissue controls (see Figure 3 in the Appendix).

Table 1: Analysis of NF-κB Binding Activity in Adjacent Tissues vs. Breast Cancer*

Sample Number	Adjacent Tissue	Tumor
1	-	++
2	-	+++
3	+/-	++
4	-	+/-
5	+/-	+++
6	-	++
7	+	++
8	+	++++
9	-	++
10	-	+

*Data shown in Table 1 were quantified by densitometry from gel mobility shifts (as shown in Figure 1). Arbitary units, from - (showing little or no binding) to ++++ to very high binding, were used to demonstrate relative quantity of binding. Examples are shown in Figure 1.

Negative finding: Classic NF-κB (p50/p65) does not appear to be activated in human breast cancer. However, our data indicate that another and potentially equally important form of NF-κB is associated with breast cancer.

Positive findings: As explained above, we have made a significant breakthrough in understanding mechanisms of oncogenesis that involve and require NF-κB. Briefly, our data indicate that NF-κB is required for many or all oncogenes to transform cells. Additionally, our data indicate that the activation of NF-κB provides protection against apoptotic cell killing, whether spontaneous (i.e., induced by oncogenes) or induced by radiological or chemotherapies. Additionally, our data indicate that NF-κB p50, p52 and c-Rel nuclear accumulation is associated with human breast cancer. Preliminary data also indicate that Bcl-3 is nuclear under these conditions. These findings allow for new approaches in establishing a role for NF-κB proteins in controlling breast cancer.

MORE EXPLICIT EXPERIMENTAL DETAILS ARE DESCRIBED IN THE FIGURE LEGENDS TO THE FIGURES PROVIDED IN THE APPENDIX.

Goals for the upcoming year (Baldwin laboratory): (1) relative to the original aim 4, we will explore whether Her2/neu activates κB-dependent transcription (as recently reported) through the targeting of the transcriptional activation domain of p65 and determine whether NF-κB is required for Her2/neu to transform cells; and (2) relative to the original aim 5, we will determine whether estrogen affects the ability of the p65 transcriptional activation domain to be functional.

Addtionally, (3) we will determine if the activation of NF-κB in breast cancer is required for oncogenic potential (using tumor model studies) and whether the activation of NF-κB in human breast cancer is functional in blocking therapeutic responses to radiation and chemotherapy. These experiments are extremely important to establish relevance for our findings. Thus, we will establish cells from breast tumors, infect with a virus encoding IκBα (the inhibitor of NF-κB) and ask if these cells or mock-infected cells can form tumors in nude mice. Additionally, we will ask whether blockage of NF-κB in breast cancers will make these cells more susceptible to different forms of cancer therapy (such as radiation and chemotherapies such as daunorubicin or camptothecin/CPT-11).

BODY (Conway Laboratory):

Summary of Gel Shifts to Evaluate Protein Interactions with the Ha-ras VNTR We have been continuing to characterize the interaction of the Ha-ras VNTR with transcription factors, including those of the NF-κB/rel family, using gel mobility shift assays. We have synthesized double-stranded 28 bp oligonucleotides corresponding to each of the 4 Ha-ras VNTR subunit repeat types carrying specific G/C polymorphisms. Oligonucleotides BstN-1 (with polymorphisms G/C), BstN-2 (C/C), BstN-3 (C/G) or BstN-4 (G/G) were ^{32}P-end-labeled and were incubated with nuclear protein extracts derived from a series of cell lines, then were run in non-denaturing polyacrylamide gels to compare the protein binding patterns among the various cell lines and to determine which repeat type was bound most tightly by nuclear proteins. Cells remained untreated or were treated (induced) with phorbol myristate acetate (PMA), phytohemagglutinin (PHA), TNF-α or a combination of PMA and PHA. Crude nuclear extracts were then prepared from a series of cell lines.

Gel shifts were conducted using the BstN-1, BstN-2, BstN-3, or BstN-4 28 bp Ha-ras VNTR probes corresponding to single repeat types 1, 2, 3, or 4, respectively. We have used a variety of breast tumor and other cell lines as sources of nuclear protein extracts in the gel shift experiments, including: MCF-7 (breast tumor), T47D (breast tumor), SKBR3 (breast tumor), BT20 (breast tumor), Jurkat (T cell leukemia), K562 (leukemia), and HeLa (cervical carcinoma). These VNTR sequences exhibited protein binding profiles that appeared to correspond to NF-κB proteins. Two bands of similar size were visible in all cell lines and binding to the VNTR sequence was the same for both the PMA-induced and the uninduced extracts. This failure by PMA induction to produce any additional protein binding suggests that the inducible form of NF-

κB, p65, does not bind the VNTR. Protein binding to the VNTR sequence was completely inhibited at both bands by the unlabeled VNTR probes, and was also inhibited by the unlabeled NF-κB (UV-21 or ab') concensus sequences, with competition strongest at the upper band (Figure 4). In addition, protein binding to the VNTR probe was inhibited by an unlabelled p53 concensus DNA-binding sequence (mainly in the lower band), but not by an SRE concensus or an Apo-B VNTR sequence. As previously shown, the AP-1 concensus sequence also competes for protein binding preferentially at the lower binding band of the Ha-ras VNTR. The results of these experiments suggest that several proteins which recognize NF-κB, AP-1 and p53 concensus DNA binding sequences also bind the Ha-ras VNTR. Because two main bands of VNTR binding occur, and each can be competed with a different sequence, there is likely more that one protein and possibly a multi-protein complex binding to the VNTR.

In order to be sure that nuclear NF-κB proteins were being induced in cells by the PMA treatments, we also performed binding experiments with a labeled NF-kB (UV-21) concensus oligonucleotide. In contrast to the Ha-ras VNTR which did not show increased protein binding in response to PMA stimulation, "classical" inducible NF-κB protein (p65) binding to the NF-κB (UV-21) sequence was observed with the PMA- and TNF-α treated extracts. The p65-DNA complex was supershifted using p65 antisera. This NF-κB binding was competed by the cold NF-κB and Ha-ras VNTR sequences. Our experiments suggest a lack of classical NF-κB/p65 binding to the VNTR; interestingly, Dr. Baldwin's lab has shown that increased NF-κB/p65 detected in most breast tumors by immunohistochemistry remains in the cytoplasm.

We have also been evaluating the potential interaction of the p53 tumor suppressor protein with the Ha-ras VNTR. The Ha-ras VNTR repeat types 3 and 4 (corresponding to the 28 bp BstN-3 and 4 oligonucleotides) possess p53 half-sites and the potential binding site lies at one of the G/C polymorphisms. P53 binds DNA in a sequence-specific manner and regulates transcription of a variety of genes (Funk et al, 1992; Friend, 1994), it is mutated or overexpressed in many breast tumors (Seth et al, 1990; Bartek et al, 1990), and, interestingly, p53 and NF-κB appear to have opposite effects on apoptosis. Dr. Baldwin's lab has recently shown that NF-κB inhibits apoptosis.

For the p53/VNTR binding studies, we have used nuclear extracts from the breast tumor lines MCF-7 (wild-type but cytoplasmic p53) and T47D (mutant p53), and oligonucleotide probes BstN-1, BstN-3 or BstN-4. We have also used the p53 consensus DNA binding sequence as a positive control in order to determine the pattern of protein binding expected with p53 for comparison with that obtained with the VNTR. The p53 and Ha-ras VNTR probes exhibited the exact same two bands of protein binding (one major and one minor), regardless of the protein extract used. These bands appeared to be the same as those seen in previous DNA binding experiments using the VNTR, AP-1 and NF-κB (UV-21) probes. There was little effect of induction by PMA on the quantity of protein bound to the p53 or the VNTR sequences.

Table 2: Summary of Gel Supershift Studies to Identify Proteins Binding to the Ha-ras VNTR

Antibody	Protein	Supershifting Observed with		
		1 repeat	2 repeats**	20 repeats***
D01(Ab1)+PAb421(Ab6)	p53 (wildtype in MCF-7 cells*)	+	+	nd
SC109 (Baldwin lab)	NF-κB/p65 (induced form)	-	ip	nd
SC114 (Baldwin lab)	NF-κB/p50 (constitutive form)	-	e	+
Ab84	NF-κB/p52 (constitutive form)	-	e	+
(Baldwin lab)	NF-κB/c-rel	-	nd	nd
SC253X, K-25	c-fos (AP-1)	-	nd	nd

ip; in progress, nd; not done, e; enhanced binding.

*p53 in MCF-7 is primarily cytoplasmic, and therefore, only a small amount of supershifting is observed presumably from contaminating cytoplasmic protein in nuclear extracts. **2 tandem repeats of the type 3 repeat.

***MVR-generated fragment corresonding to the first 20 5' repeats of an A1a common allele sequence.

NF-κB/p50 and p52 supershifts suggest these proteins bind the Ha-ras VNTR, and binding may be dependent on VNTR length. In an effort to identify the proteins which bind the Ha-ras VNTR, we have performed a series of gel supershifts using antisera specific for a number of transcriptional regulatory factors. These studies are summarized in Table 2. Supershifting using antibodies to the various NF-κB family members was performed under a number of conditions. As shown in Table 2, increasing the length of the target VNTR oligonucleotide from 1 or 2 repeat to 20 repeats permitted supershifting with antibodies to both the p50 and p52 forms of NF-κB (Figure 5). However, the very large fragment size did not yield clean results with discrete bands. In order to optimize the supershift activity with the p50 and p52 antibodies, we will evaluate binding to a series of MVR-generated fragments of increasing size. Supershifting with antibodies to p65 were initially performed only with the single repeat fragment, however, we are currently repeating this work with the longer fragments. The binding of p50 and p52 to the Ha-ras VNTR may be significant in light of the findings of Dr. Baldwin's lab showing increased nuclear localization and binding activity of p50 and p52 NF-κB in breast tumor tissue extracts.

Supershifts indicate that p53 binds the Ha-ras VNTR sequence. Some p53 antisera have been found in gel shifts to enhance or stabilize specific binding of p53 to DNA (Hupp et al, 1992). The PAb421 antibody enhances p53 sequence-specific DNA binding, and subsequent addition of the second D01 antibody may induce a supershift of this complex in gel mobility shift assays. Some investigators have also reported difficulty in super-shifting p53/DNA complexes, among them, Dr. Baldwin's lab. Nevertheless, we observed the enhanced binding of proteins to the Ha-ras VNTR sequence in the presence of the PAb421 and D01 antibodies separately, and a moderate supershift was detected with the combination of both antibodies together (Figure 6). The ability to produce a supershift did not appear to be related to VNTR length, since supershifting was detected with both the single and double repeat type 3 probes. In order to determine if p53 directly binds the Ha-ras VNTR, or requires other proteins, we have undertaken experiments with recombinant full-length wildtype p53 protein expressed in a baculovirus system (gift of Dr. Jack Griffith's lab). These studies are in progress.

Ha-ras VNTR sequence footprinting shows a 10-12 bp region of protein binding Our gel shift results indicate that a number of proteins appear to specifically bind the Ha-ras VNTR, either separately, or in a multi-protein complex. These proteins appear to be p53 and NF-κB/p50 and p52. Oligonucleotide competition studies also support the binding of these proteins, along with AP-1 factors, to the VNTR. In order to identify the regions of protein binding along the VNTR we have begun DNA footprinting experiments using the Core Footprinting System from Promega. As a target sequence for DNA footprinting, we used the same repeat type 3 doublet as that used in the gel supershift experiments. Our results indicate that there is specific protein binding to a 10-12 bp section of the 28 bp repeat within the type 3 doublet (Figure 7). We are in the process of generating a sequence ladder to run with the footprint so that we can identify the the exact bases to which the proteins bind. Examination of the 28 bp repeat sequence indicates that the most probable p53 and NF-kb binding half-sites would be positions 14-18 for p53 and 20-25 for NF-κB. Although this is yet to be determined, this stretch of 12 bp may correspond to the observed footprinting pattern.

Transcriptional enhancement by the a1, a2 and a4 common VNTR alleles depends on VNTR length. In order to evaluate potential differential transcriptional regulatory functions of the Ha-ras VNTR alleles, we have TA-cloned Ha-ras VNTR alleles (a1, a2, a4 common alleles of lengths 30, 46 and 87 repeats, respectively, and rare alleles #139 with 29 repeats, #13 with 43 repeats, and #348 with 81 repeats, into luciferase reporters downstream of both the SV-40 promoter and luciferase gene, which is the normal position of the Ha-ras VNTR with respect to the c-Ha-ras gene. The a1 and a2 common alleles are comprised of types 1, 2 and 3 repeats, while the a3 and a4 alleles also possess the type 4 repeat. Large-scale quantities of full length

a3, a4 or large rare allele/luciferase constructs have been exceedingly difficult to obtain for transfections due possibly to the instability of these sequences in bacteria or unique secondary structure which may inhibit plasmid replication. The difficulty in generating large quantities of these constructs for transfections has severely hampered our progress in this area. We have attempted a number of technical manipulations to increase our yields of these plasmids, including using SURE (rec-) bacteria for propagation of plasmids and growing bacteria with various media/conditions, however, we have been unable to solve this problem. The only way we have been able to obtain sufficient quantities of plasmids containing large alleles has been to grow up mini-prep cultures for each transfection experiment.

Transcriptional enhancement by the Ha-ras VNTR was evaluated in a number of different cell lines including the MCF-7 (normal but cytoplasmic p53), T47D (mutant p53), BT-20 (breast tumor), MCF-10 (wildtype p53, immortalized breast), and K562 (mutant p53). Transient transfections were performed as follows. Cells were plated and allowed to grow to 40-60% confluence, then were transfected with the construct of interest using Lipofectin reagent. The molar quantities of the plasmids transfected were adjusted to control for the varying lengths of the VNTR region. Cultures were allowed to grow overnight and the following morning, the medium was changed. After 48 hrs, cells were either untreated or were PMA-treated for 3 hrs. Media was changed again and the cells were harvested by lysis in 0.2 M Tris, pH 7.5 and three cycles of freezing and thawing. Cell extracts containing 10-100 ug of protein were placed in a luminometer, the substrates ATP and luciferin were simultaneously injected and light emission was measured. Luciferase expression is given in fold expression or relative light units (RLU).

As shown in Figure 8, transfection of either untreated T47D breast tumor cells or K562 cells with the Ha-ras common alleles positioned downstream from the luciferase gene produced varying levels of transcriptional enhancement above the background level of vector alone. In PMA-treated T47D, the a1 (30 repeats) or a2 (46 repeats) common VNTR alleles led to a 7.5 and 6.5-fold transcriptional, respectively, while the a4 common allele produced only a 2-fold enhancement. A similar pattern of enhancement was seen in K562 with a1, a2 and a4, however, the enhancement was higher in the untreated cells compared with the PMA-treated. The a4 common allele consistently gave the lowest level of transcriptional enhancement, however we controlled for the differences in VNTR length by adding the same molar quantities of the different luciferase constructs to the recipient cells. We are currently evaluating transfection and DNA uptake efficiency to determine if a reduced uptake of the larger a4 construct could account for the lower transcriptional enahncement observed.

The VNTR does indeed appear to possess transcriptional regulatory function, and at least a few known transcription factors may specifically bind this region. Our most important goal is to determine if the rare and common Ha-ras VNTR alleles are functionally different. We are continuing to sub-clone the a3 and a4 common alleles and a series of rare alleles, and expect that the transcriptional regulatory effects of these can be compared with the common alleles in the near future. Interestingly, the because the common alleles appear to possess some variability in enhancer function, the structural distinction between "rare/high risk" and "normal/low risk" common alleles may be more complex than we initially thought.

Goals for the upcoming year (Conway laboratory): (1) Due to the findings of Dr. Baldwin's lab indicating that breast tumor cell lines generally exhibit classical NF-κB upregulation, whereas breast tumors show a non-classical (non-p65) enhancement of NF-κB, we plan to repeat and optimize the gel shift and supershift studies using only proteins extracted from breast tumor tissue or from normal mammary epithelial cells. In fact, we will use the same tumor protein extracts as those being characterized for NF-κB in Dr. Baldwin's lab. Because of the NF-κB differences cited above, and the fact that the cell lines harbor many genetic alterations, we will discontinue our use of cell lines except for the transfection experiments. (2) In order to more clearly define the exact transcription factor binding sites, we have produced a series of mutated oligonucleotide probes for use in the gel shift and supershift studies. These experiments are underway, but we have not yet obtained results. (3) In order to identify the proteins binding the VNTR, and to determine if the binding is dependent upon the formation of a multi-protein

complex, we plan to immunoprecipitate the VNTR/protein complex(s) with antisera to p53, NF-kB and AP-1. (4) Finally, the cloning of several rare VNTR alleles has been completed, and we will evaluate their transcriptional effects in relation to the common alleles.

CONCLUSIONS (Baldwin Laboratory)

First, our data indicate that gene expression directed through NF-κB binding sites can be controlled by a previously unknown mechanism (i.e., direct targeting of low levels of constitutively active [nuclear] NF-κB) without induced nuclear translocation. This is a fundamentally important observation with wide implications. Second, our data indicate that NF-κB is required for certain (possibly all) oncogenes to neoplastically transform cells. This is a broadly significant conclusion since the inhibition of NF-κB by known inhibitors may prove useful in cancer therapy. Third, we find that NF-κB activity protects cells against apoptosis. This particular finding may explain the requirement for NF-κB in transformation since protection against apoptosis is likely to be a critical component of oncogenesis. Additionally, resistance of tumors to radiation or chemotherapy may be based on the activation of NF-κB by these stimuli (thus blunting the therapeutic potential of these therapies). Finally, we now show that an unexpected form(s) of NF-κB is activated in human breast cancer. Each of these finding is fundamentally important to our understanding of cancer, and are likely to be essential for the development and progression of breast cancer. These data provide an important new foundation for pursuing our original aims and provide hope for developing a new generation of cancer therapies based on the inhibition of NF-κB.

CONCLUSIONS (Conway Laboratory)

Identification of p53 and NF-κB/p50 and p52 proteins binding the Ha-ras VNTR Our gel-shift and supershift studies indicate that several proteins bind the Ha-ras VNTR and this binding is observed as two distinct bands on the gels. Oligonucleotide competition suggested that NF-κB proteins, AP-1 proteins and p53 might be involved in the binding. A series of gel supershift experiments were performed using antibodies specific for members of the NF-κB protein family (p50, p52, p65 and c-rel), AP-1 binding proteins (c-fos, c-jun) and p53. These studies have demonstrated that p53, and the constitutive NF-κB proteins p50 and p52 bind the VNTR, and this binding may occur preferentially along multiple tandem 28 bp repeats, as opposed to single repeats. The NF-κB supershifting was only observed with the longer fragments. Footprinting of the VNTR sequence showing a 10-12 bp region of protein binding supports the gel shift studies indicating that proteins specifically bind the VNTR.

Our transient transfection experiments demonstrate that the Ha-ras VNTR does possess a transcriptional regulatory function. The transcriptional effects appear to be related to VNTR length (and/or sequence) in that the common alleles a1, a2 and a4 produce varying levels of transcriptional enhancement. The exact effects also appear to be cell line-specific and are influenced by PMA-stimulation of the transfected cells. The most important question is whether the rare alleles possess a different function. These results are potentially very important because if the VNTR possesses a biological function, it may be a direct participant in the carcinogenic process.

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APPENDIX (Baldwin Laboratory)

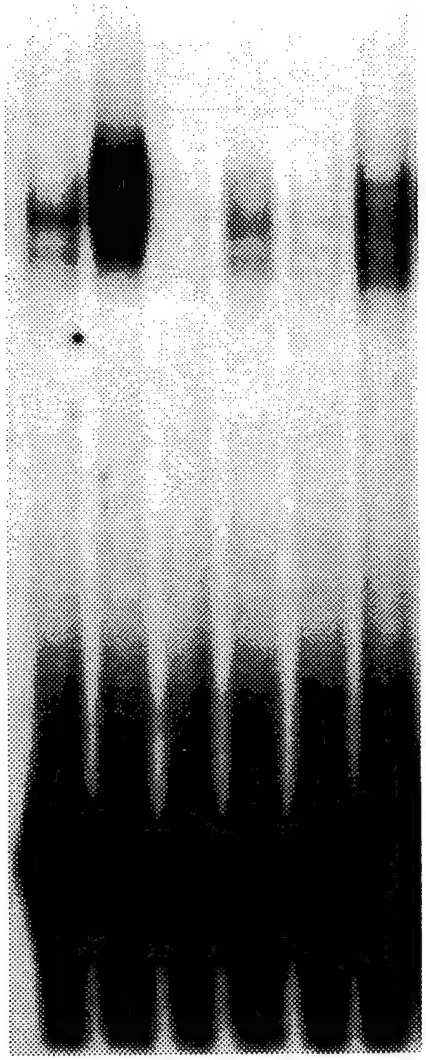


Figure 1. Human Breast Cancers Exhibit Induced Nuclear Accumulation of an NF-κB Binding Activity. Nuclear extracts were prepared from human breast tumors (Provided by the Lineberger Cancer Center Tumor Procurement Facility) and matched adjacent non-tumor tissue. 5 μg of nuclear extract was incubated with a ^{32}P-radiolabeled class I MHC NF-κB binding site probe and electrophoresed on a 5% polyacrylamide gel. The figure shows the free probe at the bottom and the complexes that are formed. Lanes 1, 3, and 5 are from adjacent tissue while lanes 2, 4 and 6 are from the corresponding tumor samples. Lanes 1,2 and 3,4 and 5,6 are from the same tumor samples respectively. Lanes 1,2 correspond to tumor sample #8 (from Table 1); lanes 3,4 correspond to sample #10; and lanes 5,6 correspond to tumor #1.

Figure 2. Supershift experiments indicate that both p50 and p52 are activated in human breast cancer. Experiments were performed as in Figure 1 (using human breast cancer extracts) but antibodies to NF-κB1 (p50) or NF-κB2 (p52) (obtained from Rockland, Inc.) were incubated with extracts prior to incubation with the radiolabeled NF-κB probe. Supershifted complexes are shown for the p50 antibody (lane 3) and for the p52 antibody (lane 4). Lane 1 is the resulting gel mobility shift complex obtained with nuclear extracts from adjacent tissue and Lane 2 is the human breast cancer extracts.

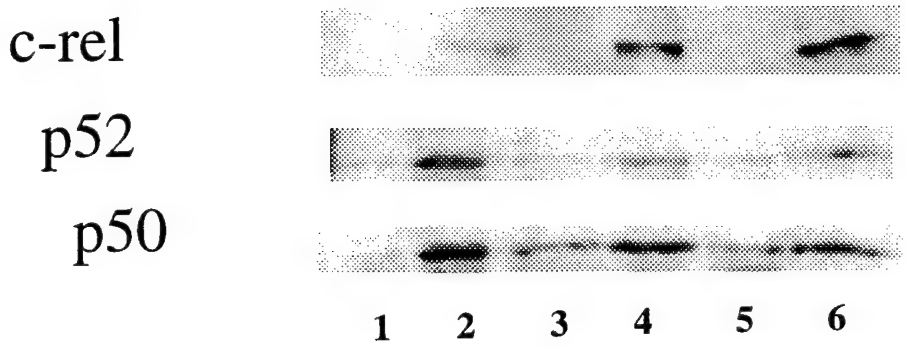


Figure 3. Western blotting confirms the increase in nuclear p50 and p52 along with an increase in c-Rel. Nuclear extracts (as described above) from matched adjacent tissue/tumors were electrophoresed on denaturing polyacrylamide gels, transferred to membrane, and probed with antibodies to p50, p52 and c-Rel. Lanes 1, 3 and 5 are from adjacent tissues and lanes 2, 4 and 6 from the corresponding, matched tumors.

APPENDIX (Conway Laboratory)

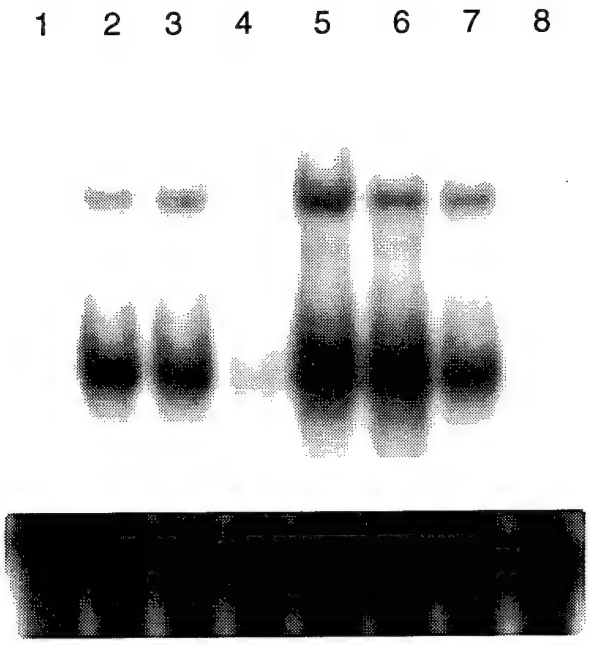


Figure 4: Gel shift showing protein binding to the Ha-ras VNTR sequence and competition for binding by other sequences. *Lane 1*; Ha-ras VNTR type 3 repeat doublet probe only, *lane 2*; with nuclear protein extract from uninduced MCF-7 cells, *lane 3*; with extract from TNF-α induced MCF-7, *lane 4*; competition for binding by NF-κB oligonucleotide (ab'), *lane 5*; induced MCF-7 extract plus NF-κB/p50 antisera, *lane 6*; induced MCF-7 extract plus NF-κB/p52 antisera, *lane 7*; lack of competition by SRE oligonucleotide, *lane 8*; competition by unlabeled VNTR probe.

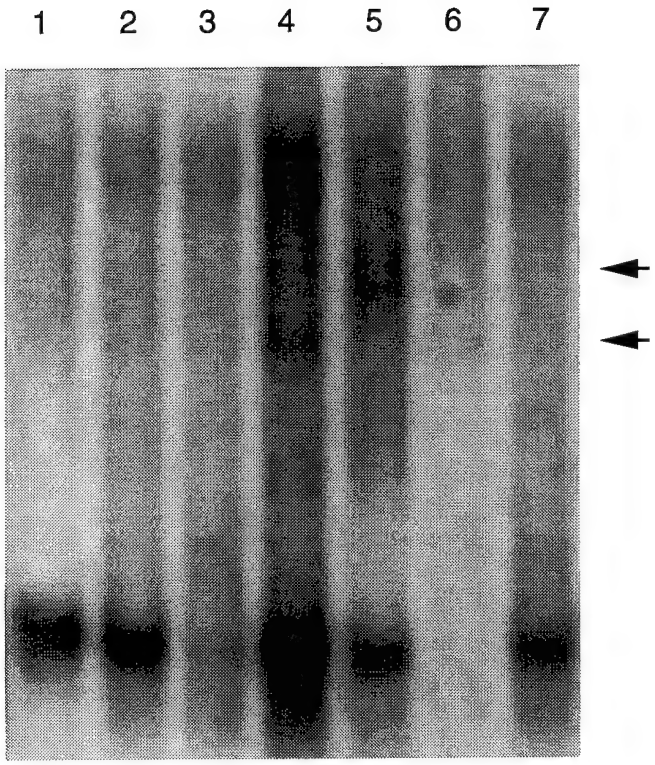


Figure 5: Gel supershift showing NF-kB/p50 and p52 protein binding to the Ha-ras VNTR 20-repeat MVR probe. *Lane 1*; uninduced MCF-7 nuclear extract, *lane 2*; PMA-induced MCF-7 nuclear extract, *lane 3*; competition for binding by NF-kB oligonucleotide (ab'), *lane 4*; supershift with addition of NF-kB/p50 antisera (indicated by arrow), *lane 5*; supershift with addition of NF-kB/p52 antisera (indicated by arrow), *lane 6*; competition for binding by unlabeled Ha-ras VNTR 20-repeat probe, *lane 7*; lack of competition for binding by ApoB VNTR oligonucleotide.

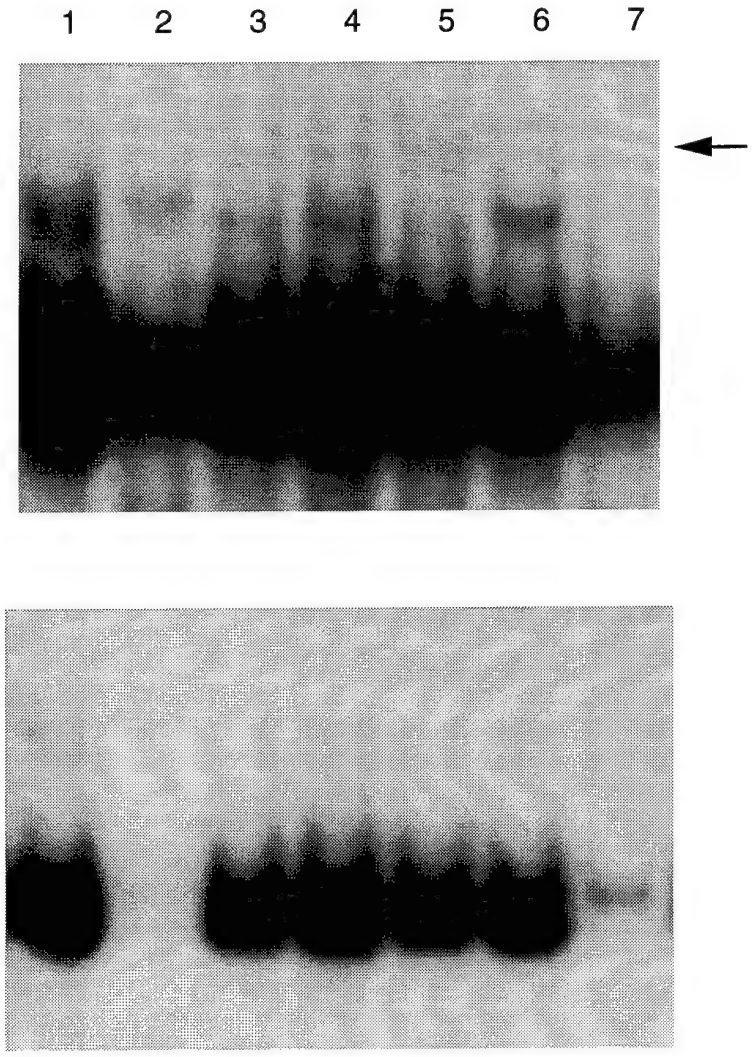


Figure 6: Gel supershift showing p53 binding to the Ha-ras VNTR repeat type 3 (BstN3) probe. Two different exposures of the same gel are given; the longer exposure of the top panel shows the supershift. *Lane 1*; BstN3 probe only, *lane 2*; uninduced MCF-7 nuclear extract, *lane 3*; competition by p53 oligonucleotide, *lane 4*; enhanced binding to BstN3 by addition of p53 Ab-1 antisera, *lane 5*; enhanced binding to BstN3 by addition of p53 Ab-6 antisera, *lane 6*; supershift with both Ab-1 and Ab-6 antisera together (indicated by arrow), *lane 7*; lack of competition for binding by SRE oligonucleotide.

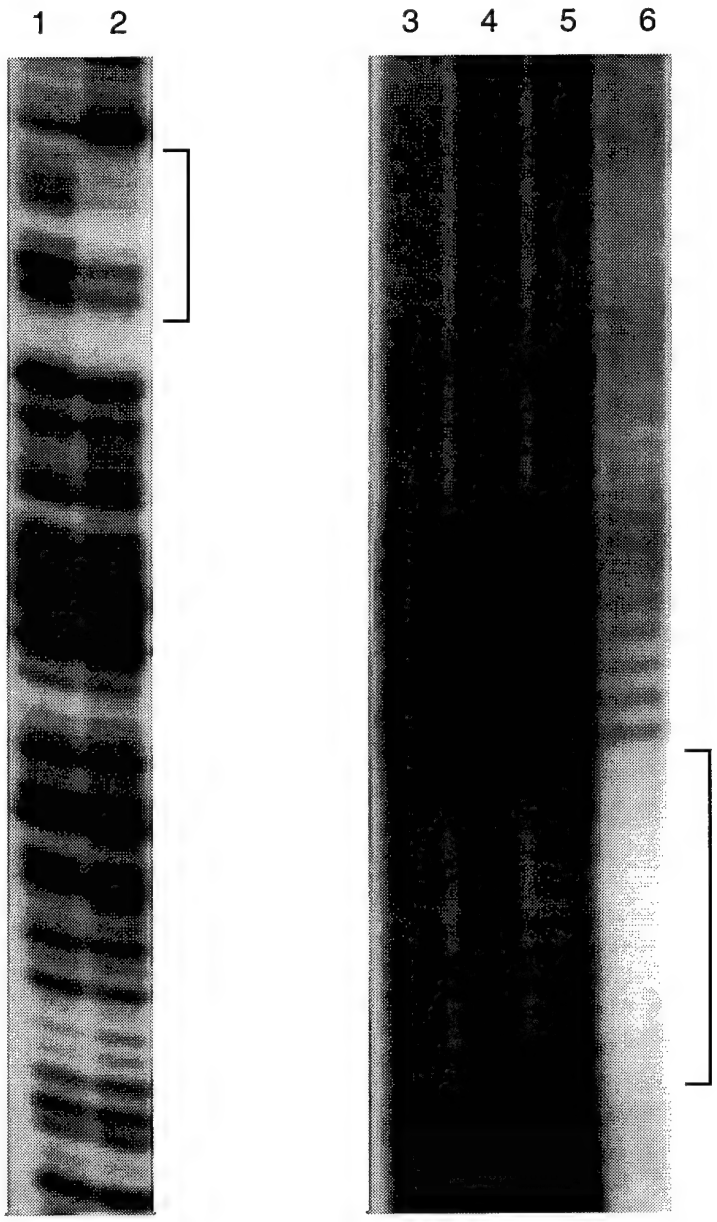
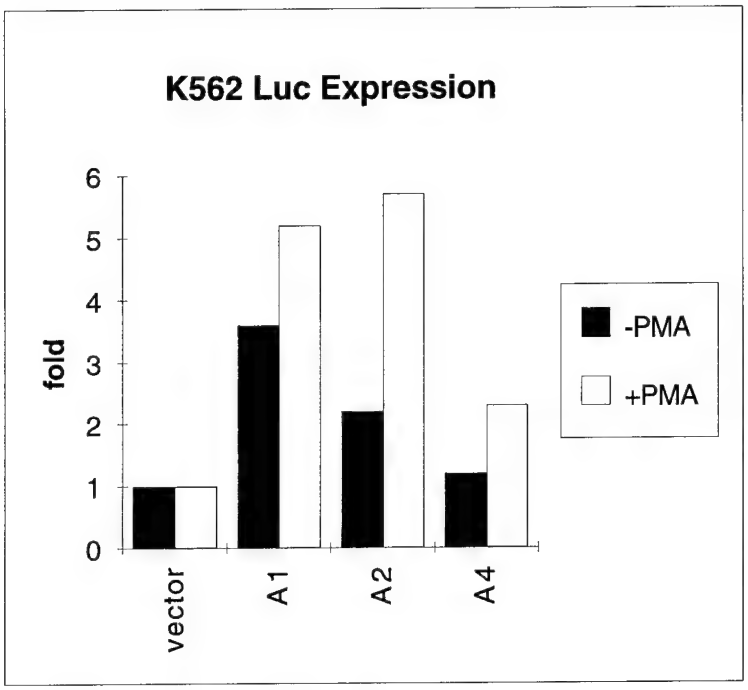


Figure 7: Preliminary DNAse I footprinting of the Ha-ras VNTR repeat type 3 doublet sequence. *Lane 1*; SV40 control DNA only, *lane 2*; SV40 positive control DNA plus 1 ul AP-2 extract (included in Promega kit), *lane 3*; Ha-ras VNTR sequence only, *lane 4*; VNTR plus 1 ul MCF-7 nuclear extract, *lane 5*; VNTR plus 5 ul MCF-7 nuclear extract, *lane 6*; VNTR plus 10 ul MCF-7 nuclear extract showing footprinted region near bottom of gel. Footprinted sequences in the control and the VNTR sequence (10-12 bp) are indicated by brackets.

A:



B:

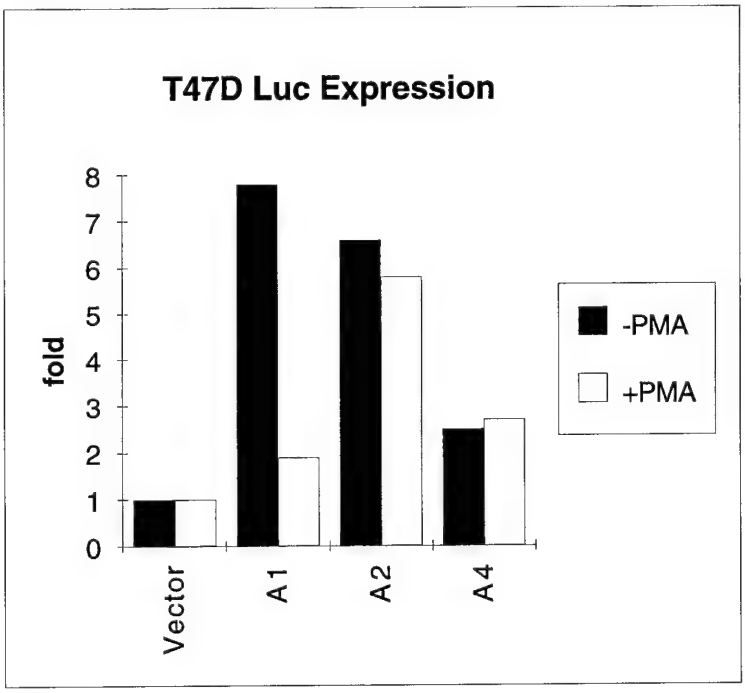


Figure 8: Transcriptional regulatory effects of the Ha-ras VNTR a1, a2 and a4 common alleles in uninduced or PMA-treated K562 or T47D cell lines. A; luciferase expression in K562, B; luciferase expression in T47D. Luciferase expression is given in fold enhancement.

Final Draft
(in revision, Science)

Cellular Transformation by Oncogenic Ras: Requirement of NF-κB Activation to Suppress p53-Independent Apoptosis

Marty W. Mayo, Cun-Yu Wang @^, Patricia C. Cogswell, Kelley S. Rogers-Graham ϕ, Scott W. Lowe+, Channing J. Der @ϕ, and Albert S. Baldwin Jr. #@$

Lineberger Comprehensive Cancer, # Department of Biology, and @Curriculum in Genetics and Molecular Biology, ^Department of Endodontics of the School of Dentistry, ϕDepartment of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7295
and +Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724.

$To whom correspondence should be addressed at:
Lineberger Comprehensive Cancer Center,
Campus Box 7295,
University of North Carolina,
Chapel Hill, NC 27599, USA

Phone: (919) 966-3652
Fax: (919) 966-0444

Abstract:

ras is a widely mutated proto-oncogene in human tumors that functions to chronically stimulate signal transduction cascades resulting in the induction or activation of specific transcription factors, including Ets, c-Myc, c-Jun and NF-κB. Although it has been demonstrated that these Ras-responsive transcription factors are required for transformation, the mechanisms by which these proteins facilitate oncogenesis have not been established. We demonstrate here that oncogenic Ras requires the activation of NF-κB to protect cells from undergoing transformation-induced programmed cell death. Importantly, apoptosis induced by oncogenic Ras following the inactivation of NF-κB is mediated by a p53-independent mechanism. Our results provide an explanation for the requirement of NF-κB for Ras-mediated oncogenesis and provide a therapeutic rationale for tumor therapy regardless of the functional status of the p53 tumor suppressor gene product.

Mutations in a *ras* allele occur in 30% of all human tumors (1), making *ras* the most widely mutated human proto-oncogene. Significant progress has been made towards elucidating both the signal transduction pathways and the transcription factors that are involved in Ras signalling (1). Thus, it has been shown that both Raf/MEK/Erk-dependent and -independent pathways control Ras-induced cellular responses (2) and that these signal transduction pathways ultimately control the ability of this oncoprotein to activate downstream transcription factors (3). It has been demonstrated that Ets, Myc and c-Jun proteins are Ras-responsive transcription factors required for cellular transformation *in vitro* (4) and *in vivo* (5). We have previously shown that, like Ets, Myc and c-Jun, NF-κB activation is downstream of Ras and that Ras stimulates transcription directed by NF-κB binding sites (6). In addition, we have recently shown that Ras activates NF-κB largely through the stimulation of the transcriptional activation function of the NF-κB RelA/p65 subunit and that Ras requires NF-κB for focus-forming activity (7). However, the molecular mechanisms by which NF-κB and other transcription factors facilitate oncogenesis have not been clearly established. In this study we report that oncogenic Ras requires NF-κB activation to block transformation-induced programmed cell death.

It was recently reported by our group (8) and by others (9) that the activation of NF-κB provided protection against apoptosis initiated by tumor necrosis factor, transforming growth factor-β1 and several cancer therapies, such as radiation and chemotherapy. Based on the demonstrations that NF-κB-dependent gene expression is targeted by oncogenic Ras (6) and that NF-κB may play a direct role in cellular transformation (reviewed in 10) and on the evidence that oncogenesis may require an anti-apoptotic function (11), we were interested in determining whether the requirement for NF-κB in mediating Ras transformation was through a cell survival function. To address this question, we inhibited NF-κB activity by utilizing the super-repressor form of IκBα, which contains serine-to-alanine mutations at residues 32 and 36. This highly specific inhibitor of NF-κB cannot be phosphorylated (12) or degraded through a proteasome-dependent pathway (13) and, therefore, blocks the nuclear accumulation of NF-κB and the subsequent transactivation of κB-responsive genes (8). To determine whether the inhibition of NF-κB in Ras-transformed cells would initiate a cell death response, we first utilized β-galactosidase expression assays to measure cell viability. Parental NIH3T3 and H-

Ras-transformed cells (3T3 H-Ras) were cotransfected with a pCMV-LacZ reporter and with either an empty expression vector control or with the vector encoding super-repressor IκBα. As shown in Fig. 1, H-Ras-transformed NIH3T3 cells co-expressing the super-repressor IκBα displayed a marked decrease in the total number of β-galactosidase-positive cells as compared to cells transfected with the vector control. In contrast, parental NIH3T3 cells expressing the super-repressor IκBα did not display a reduction in the number of β-galactosidase positive cells. These results indicate that the expression from the pCMV-lacZ reporter was not affected by the super-repressor and that expression of IκBα did not kill non-transformed parental cells (Fig. 1). Similar results to those shown in Fig. 1 were obtained with H-Ras(V12)-transformed Rat-1 fibroblasts and C127 mouse mammary epithelial cells, indicating that the killing observed in 3T3 H-Ras cells following the expression of the super-repressor IκBα was not a cell type specific phenomenon (14). Since a gain of function mutation in either K- or N-*ras* is more prevalent in human cancers than mutations in H-*ras* (1), we wanted to determine if NF-κB served a similar protective role for other oncogenic Ras proteins. Like 3T3 H-Ras cells, NIH3T3 cells transformed with activated alleles of *ras* (namely, K-, N-, and R-) were susceptible to killing following the expression of the super-repressor IκBα (Fig. 1). Importantly, for control purposes we demonstrated that parental NIH3T3 cells cotransfected with wild-type H-Ras did not result in a loss of cell viability in the presence of the super-repressor IκBα (14). Furthermore, cotransfection experiments with the super-repressor IκBα and c-Ets, an oncogene which is known to weakly transform NIH3T3 cells (15), also did not result in a significant loss in β-galactosidase positive cell number (14). Thus, these results indicate that oncogenic Ras (but not c-Ets) requires the cell survival function of NF-κB to overcome the activation of a death pathway initiated in transformed cells.

To establish whether the loss of cell viability was a direct result of oncogenic Ras and was not due to secondary mutations which might have occurred after the generation of the 3T3 H-Ras cell line, we utilized the Rat-1:iRas cell line, which contains a stably integrated oncogenic H-*ras*(V12) gene under the controlled expression of an IPTG-inducible promoter (16). To inhibit NF-κB activation, we established the Rat-1:iRasI line, which constitutively expresses the super-repressor IκBα. Additionally, we established a control line, Rat-

1:iRasV, which contains the empty expression vector. Following IPTG addition the Rat-1:iRasV and I clones displayed similar levels of $p21^{ras}$ protein compared to the parental Rat-1:iRas cells (Fig. 2A). Immunoblotting with IκBα-specific antibody detected the endogenous IκBα protein while Rat-1:iRasI cells also displayed a larger immunoreactive band which corresponded to the super-repressor IκBα (Fig. 2A). Interestingly, the IPTG-induced expression of oncogenic H-Ras in Rat-1:iRasV cells resulted in a modest increase in NF-κB DNA-binding (Fig. 2B, compare lanes 1 and 2) and in κB-dependent transcription (Fig. 2C). The increase in NF-κB DNA-binding following the inducible expression of $p21^{ras}$ indicates that this oncoprotein can induce nuclear translocation of NF-κB. However, this concept is different in an established Ras-transformed setting (such as 3T3 H-Ras cells) in which constitutive expression of oncogenic Ras predominantly regulates RelA/p65 through a transactivation function and not through nuclear translocation (7). As predicted by the specificity of the super-repressor IκBα, nuclear extracts isolated from the Rat-1:iRasI line did not demonstrate NF-κB-binding activity (Fig. 2B) or an increase in transactivation of the 3x-κB-dependent reporter (Fig. 2C) following IPTG addition. Furthermore, compared to the control line, Rat-1:iRasI cells failed to demonstrate TNFα-induced nuclear translocation of NF-κB as determined by EMSAs (Fig. 2B). To ensure that clonal differences had not occurred during the selection process, five individual puromycin-resistant clones, expressing similar levels of the IκBα transgene, were pooled (Rat-1:iRasI-Pool) and analyzed. The Rat-1:iRasI-Pool demonstrated virtually identical results to those obtained using individual Rat-1:iRasI clones (14). These results demonstrate that the constitutive expression of the super-repressor IκBα in the Rat-1:iRasI cells effectively inhibits NF-κB DNA-binding and κB-dependent transcription following cellular stimulation induced by either oncogenic Ras or TNFα.

To determine if the induction of Ras leads to a loss of cell viability under conditions where NF-κB is inhibited, Rat-1:iRasI and control cells were subjected to IPTG treatment and total cell numbers were examined over a three day period. A significant reduction in cell viability was observed in the Rat-1:iRasI cells following Ras expression, compared to the vector control cells (Fig. 3A). It should be noted that in the absence of IPTG there is no appreciable difference in growth rates or survivability of the different cells (14). To address whether

this loss in cell viability was due to oncogenic Ras inducing apoptosis following the inactivation of NF-κB, Rat-1:iRasI and control cells were analyzed for DNA strand breaks by performing both the deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay and agarose gel electrophoresis. The induction of activated Ras in Rat-1:iRasI cells resulted in an increase in genomic DNA fragmentation evident 36 hours post IPTG addition (14). IPTG treatment for 48 hours resulted in the dramatic appearance of TUNEL positive cells in the Rat-1:iRasI line with virtually no TUNEL positive uninduced cells or vector control cells (Fig. 3B). Additionally, the Rat-1:iRasV and I cells displayed distinct Ras-transformed morphology following IPTG induction (Fig. 3B and see ref. 16). Furthermore, FACS analysis of propidium iodine and BrDU stained cells confirmed that the IPTG induction did not induce senescence and that the <2N DNA peak represented apoptotic cells and not necrotic cells (14). Thus, the induction of oncogenic Ras expression in the presence of a specific inhibitor of NF-κB leads to pronounced enhancement of apoptosis. Our data strongly supports the requirement of NF-κB for the survival of oncogenic Ras-expressing cells, however not all of the Rat-1:iRasI cells undergo programmed cell death following H-Ras (V12) expression, but instead a subpopulation of cells survive (Fig. 3A). These findings may be explained by the possibility that oncogenic Ras activates cell survival factors in addition to NF-κB or they may be explained by our observations that prolonged exposure of Rat-1:iRasI cells to IPTG leads to a loss of oncogenic H-Ras expression without a reduction in super-repressor IκBα protein or function (14). Collectively, our results strongly support our hypothesis that oncogenic Ras requires NF-κB to block transformation-induced programmed cell death thereby maintaining a cellular transformed state.

Since oncogenic Ras induces apoptosis following the inactivation of NF-κB and because the tumor suppressor p53 mediates a variety of apoptotic events (reviewed in 18), we were interested in determining whether the Ras-mediated killing required the p53 tumor suppressor gene product. To address this issue we utilized mouse embryo fibroblasts (MEFs) isolated from $p53^{-/-}$ deficient mice (17). Data presented in Fig. 1 suggested to us that oncogenes that activate NF-κB may also require this transcription factor for a cell survival function. For this reason, we utilized $p53^{-/-}$ MEFs harboring either E1A, an oncogene which is known to activate NF-κB (19), or E1A plus H-Ras. As shown in Figure 4A, $p53^{-/-}$ MEFs containing E1A or both E1A and H-Ras displayed a significant reduction in the total number of β-galactosidase positive cells following co-

expression of the super-repressor IκBα. In contrast, $p53^{-/-}$ MEFs alone did not exhibit a reduction in the percent of β-galactosidase positive cell staining following the expression of the super-repressor (Fig. 4A). Although E1A induces killing in a p53-dependent manner (20), our data indicate that in a $p53^{-/-}$ deficient background the E1A oncogene requires the protective function of NF-κB. To confirm that E1A was not required for Ras-mediated killing, $p53^{-/-}$ MEF cells were cotransfected with pCMV-LacZ, H-Ras(V12) and with either the empty expression vector or the super-repressor IκBα. As shown in Fig. 4B, $p53^{-/-}$ MEFs cotransfected with oncogenic H-Ras, but not wild-type H-Ras, were effectively killed following the inactivation of NF-κB by the super-repressor IκBα. Therefore, these data indicate that the p53 tumor suppressor gene product is not required for the induction of apoptosis in response to oncogenic Ras-transformation when NF-κB activity is blocked.

Our data strongly support the hypothesis that Ras oncogenes require an anti-apoptotic function in order for transformed cells to survive. The data shown here indicate that oncogenic Ras elicits both pro-apoptotic and anti-apoptotic pathways, with the latter pathway being dominant in immortalized cells. Although oncogenic Ras can induce senescence in primary cells (21), as well as programmed cell death (22), Ras has also been demonstrated to suppress apoptosis induced by Myc and E1A (22,23). Thus the recent report (23) that Ras can both induce and inhibit apoptosis is completely consistent with our results. In that study, the activation of PI(3) kinase by Ras was shown to suppress c-Myc induced apoptosis through the activation of the kinase PKB/Akt. Additionally, other reports have identified the cell survival function of PKB/Akt (24). Thus, it will be important to determine if PKB/Akt is involved with Ras-induced NF-κB activation. Our data also indicate that oncogenes which activate NF-κB (such as Ras and E1A) utilize the anti-apoptotic potential of this transcription factor. Therefore, the ability of Ras to utilize the anti-apoptotic potential of NF-κB may have broader implications in human cancer since many oncogenic tyrosine kinases induce their proliferative signals through Ras-dependent pathways (reviewed in 25) and since several of these kinases are known to activate NF-κB.

Data shown in Fig. 4 demonstrate that cell killing mediated by Ras (in the absence of NF-κB) occurs through a p53-independent mechanism. This point is further supported by our observations that cell culture lines which constitutively display nuclear NF-κB and elevated κB-dependent reporter gene expression and which lack functional p53 expression (including HeLa, HEP3B, and SAOS-2) are killed by expression of the

super-repressor form of IκBα (14). Our findings are also supported by a recent study in which it was demonstrated that the status of p53 was not a contributing factor in the induction of apoptosis in H-Ras-mediated development of mammary and salivary tumors *in vivo* (26).

These studies have important implications relative to cancer treatment. First, our data indicate that tumors driven by mutant alleles of *ras* may be sensitive to killing by functional inhibitors of NF-κB. It is important to note that constitutive oncogenic Ras activates NF-κB largely through the signal pathway induced stimulation of the transcriptional activation domains of the RelA/p65 subunit (7). Thus, development of potential inhibitors should be focused on the difference between blocking the induced nuclear translocation of NF-κB and inhibiting the activation of transcriptional function. Another issue relates to our observations that not all tumor cell lines expressing oncogenic Ras are sensitive to killing by inhibitors of NF-κB, suggesting that other oncogenes expressed in these cells may provide κB-independent cell survival functions. This idea is supported by the fact that the ets transcription factors are known to be activated by Ras (see discussion above), that wild-type ets can serve a cell survival function (27) and that ets-expressing cells are not killed by the super-repressor form of IκB. Another important issue relative to tumor treatment is our observation that the Ras-induced cell killing is p53 independent. This is potentially a very important point, since greater than 90% of late-stage metastatic tumors fail to express a functional p53 tumor suppressor gene product, which most likely accounts for the inability of some genotoxic agents to initiate programmed cell death in these tumor cells (17, 26). Thus, our data raises the possibility that NF-κB inhibitors may serve as a primary therapy for certain Ras- or other oncogene-driven tumors that would not be dependent on the status of the p53 tumor suppressor gene product.

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Figure Legends:

Fig. 1. Expression of the super-repressor IκBα in Ras-transformed NIH3T3 cells induces cell killing. Parental NIH3T3, Ras-transformed NIH3T3 cells were cotransfected with pCMV-LacZ (pcDNA3-lacZ, Invitrogen, 0.2 μg/24 well plate) and either an empty expression vector control or with the super-repressor IκBα (SR-IκBα, 1μg/ plate, each) using lipofectamine (Gibico/BRL). Forty-eight hours following the start of transfection, cells were fixed, stained with X-Gal and β-galactosidase expressing cells were numerated in each of 24 wells. These results represent the mean ± SD of three independent experiments performed in triplicate.

Fig. 2. A Rat-1 cell line, containing inducible oncogenic vH-Ras, displays a loss of NF-κB activity following the expression of the super-represssor IκBα. (**A**). Generation and characterization of the Rat-1:iRas cell line harboring the super-repressor IκBα. Rat-1:iRas cells, containing an IPTG-inducible H-Ras(V12) gene (16), were cotransfected with the pCMV-4 empty expression vector or with pCMV-4 containing a cDNA encoding the super-repressor IκBα (1μg each) and with the pBabe-puro (0.2 μg) which provided puromycin resistance at a concentration of 1μg/ml. The Rat-1:iRasV line contains the empty expression vector, while Rat-1:iRasI line expresses the super-repressor. Parental Rat-1:iRas, and V and I cells were treated with 5mM IPTG for 24 hours and protein expression was assessed by western blotting total cell extracts (60 μg/lane) with either a pan-Ras antibody (Ab-4, Cat# OP41, Calbiochem) or with IκBα specific antibody (C-21, Cat# SC-371, Santa Cruz) and detected by ECL (Amersham). Immunoblotting performed with IκBα antibody displayed a faster migrating protein (35 K) which corresponds to the endogenous Rat IκBα, while the Rat-1:iRasI cells also displayed a slower migrating immunoreactive band (37K) which corresponds to the super-repressor IκBα protein. (**B and C**). Expression of the super repressor IκBα in Rat-1:iRasI cells effectively blocked both TNFα-stimulated and Ras-mediated NF-κB nuclear translocation as well as κB-dependent gene expression. Nuclear extracts (5 μg each), isolated from Rat-1:iRasV and I cells in either the absence or presence of IPTG (5 mM for 16 hours) or following TNFα addition (10 ng/ml for 15 minitues), were analyzed by electrophoretic mobility shift assays using (EMSAs) using a [^{32}P]-labeled high affinity NF-κB consensus and were performed as previously

described (8). Rat-1:iRasV and I cells were cotransfected with the pcDNA3-lacZ (2 μg) or with either 3x-mut κB-LUC or 3x-κB-LUC reporter (2 μg each) as described in Fig. 1. Twenty-four hours following transfection cells were washed and media containing reduced serum (1%) either lacking or containing 5 mM IPTG was added. Cell extracts were harvested 12 hours later and luciferase activities were measured and normalized to the level of β-galactosidase activity. The results presented are the average of two independent transfection experiments performed in triplicate.

Fig.3. Oncogenic Ras requires NF-κB activation to inhibit apoptosis. (**A**). Rat-1:iRasI and control cells ($1x10^6$) were plated into 100mm dishes containing complete DMEM media supplemented with 10% FCS and 12 hours later cells were stimulated in either the absence or presence of 5mM IPTG. Cells were harvested over the time course indicated and total cell numbers and viabilities were determined by trypan blue dye exclusion. The data presented represents the mean (SD $\leq$ 2.7) of three independent experiments. The relative cell number was calculated as the total number of cells following IPTG addition divided the unstimulated cell count (which was normalized to 100%). (**B**) Rat-1:iRasV and I cells were either left untreated or were subjected to IPTG (5 mM) for 48 hours and then fixed with 4% papa formaldehyde and TUNEL staining was performed according to the manufactor's protocol (Boehringer Mannheim). TUNEL positive cells (indicated by reddish/brown stain) display membrane blebbing and condensed morphology (characteristics typical of cells under going apoptosis).

Fig. 4. Oncogenic Ras-mediates p53-independent programmed cell death in the absence of NF-κB. (**A**). $p53^{-/-}$ MEFs (passage 5) and $p53^{-/-}$ MEFs transformed with either E1A or E1A plus activated H-Ras(V12) were cotransfected with the pcDNA-lacZ (0.2 μg) and with the empty expression vector, or with the super-repressor IκBα (1μg each/ 24 well plate). Forty-eight hours following the start of transfection cells were fixed, stained and β-galactosidase expressing cells were numerated. This data represents three-independent experiments performed in triplicate. (**B**). $p53^{-/-}$ MEFs (passage 5) were cotransfected with the pcDNA-lacZ (0.2 μg/ 24 well plate) along with either the empty expression vector control, or with various expression constructs indicated

(μg/ 24 well plate). Cells were stained for β-galactosidase activity 48 hours post-transfection and the mean ± SD represents two experiments performed in triplicate.

Figure 1

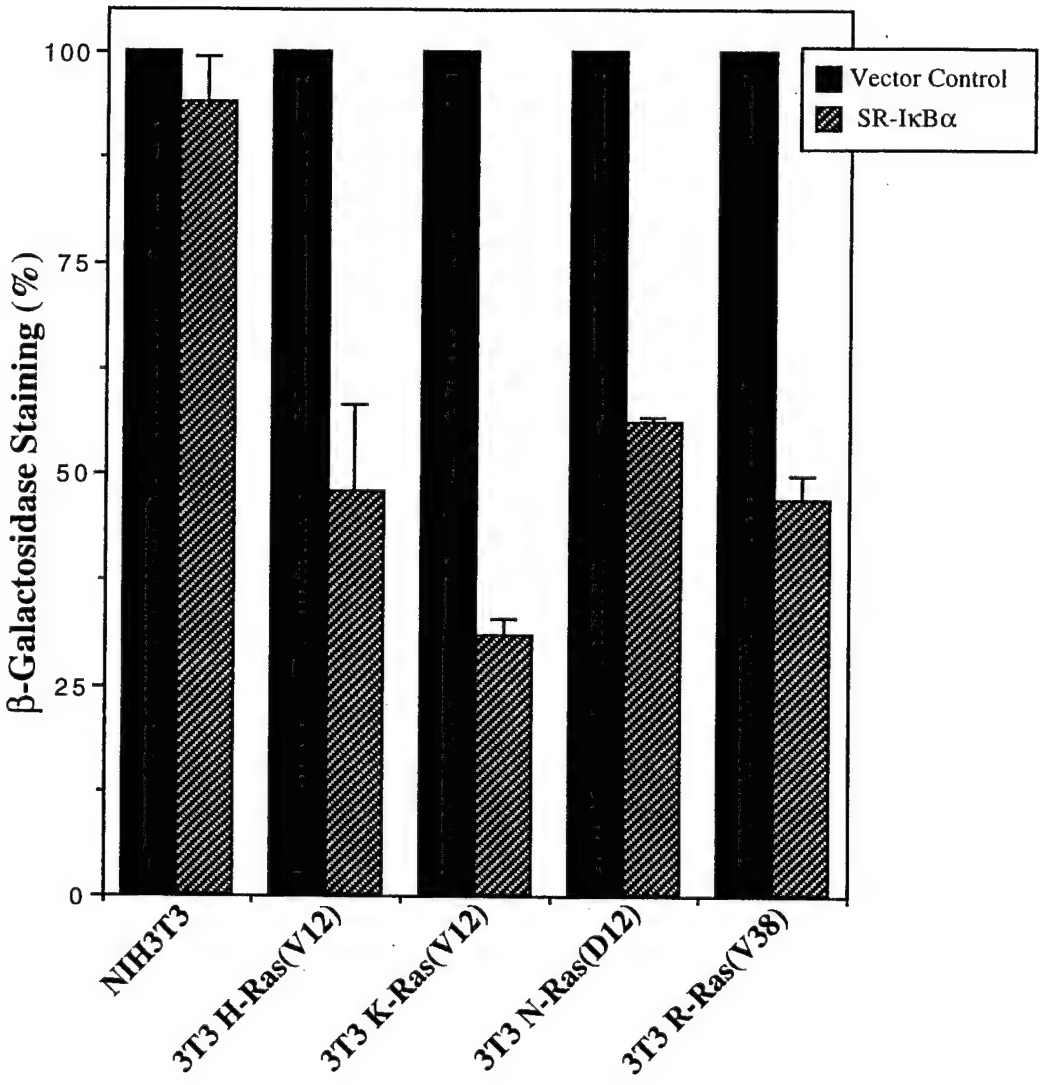

β-Galactosidase Staining (%)
100
75
50
25
0
Vector Control
SR-IκBα
NIH3T3
3T3 H-Ras(V12)
3T3 K-Ras(V12)
3T3 N-Ras(D12)
3T3 R-Ras(V38)

Figure 2A

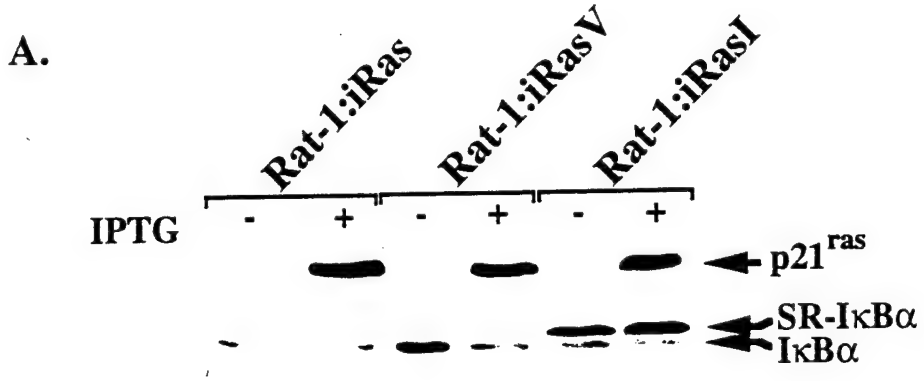


Figure 2B

B.

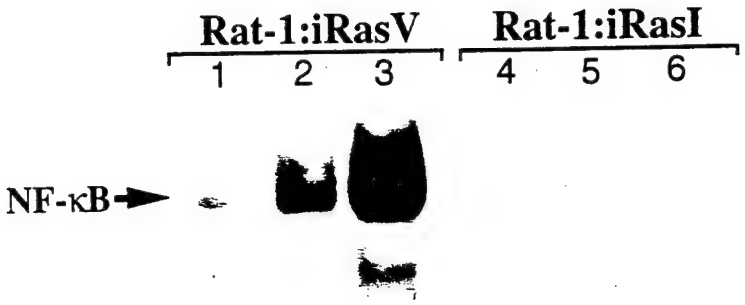


Figure 2C

C.

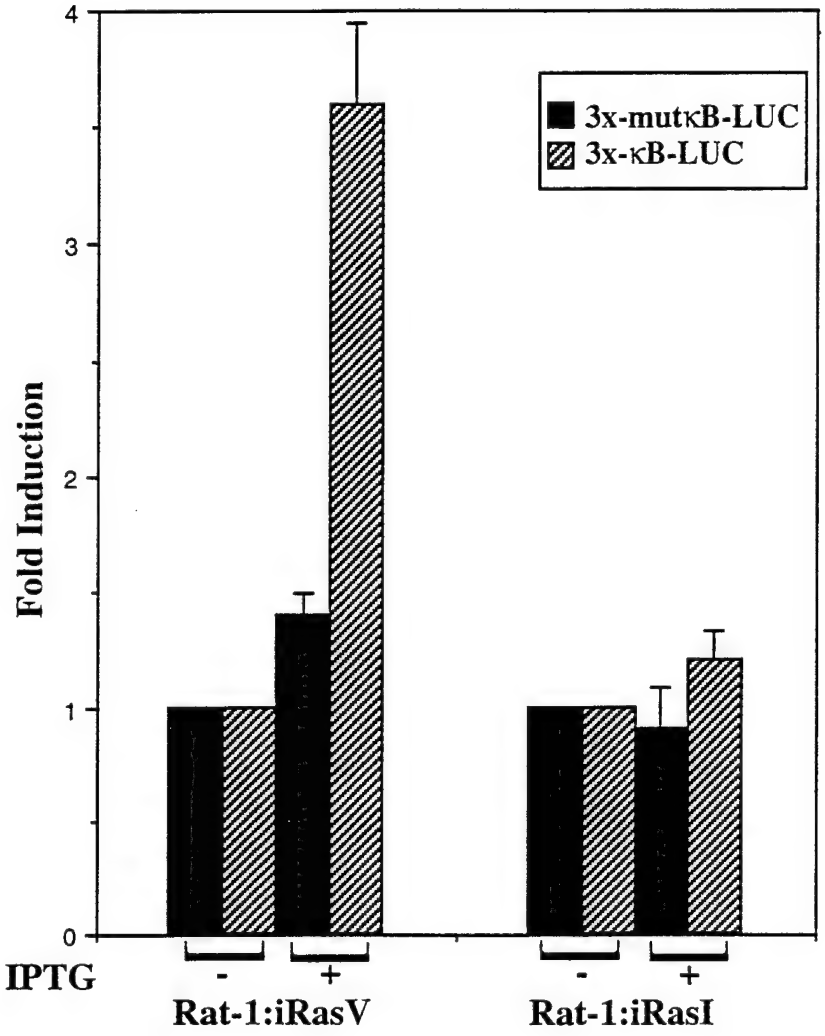

4
3
2
1
0
Fold Induction
3x-mutκB-LUC
3x-κB-LUC
IPTG
-
+
-
+
Rat-1:iRasV
Rat-1:iRasI

Figure 3A

A.

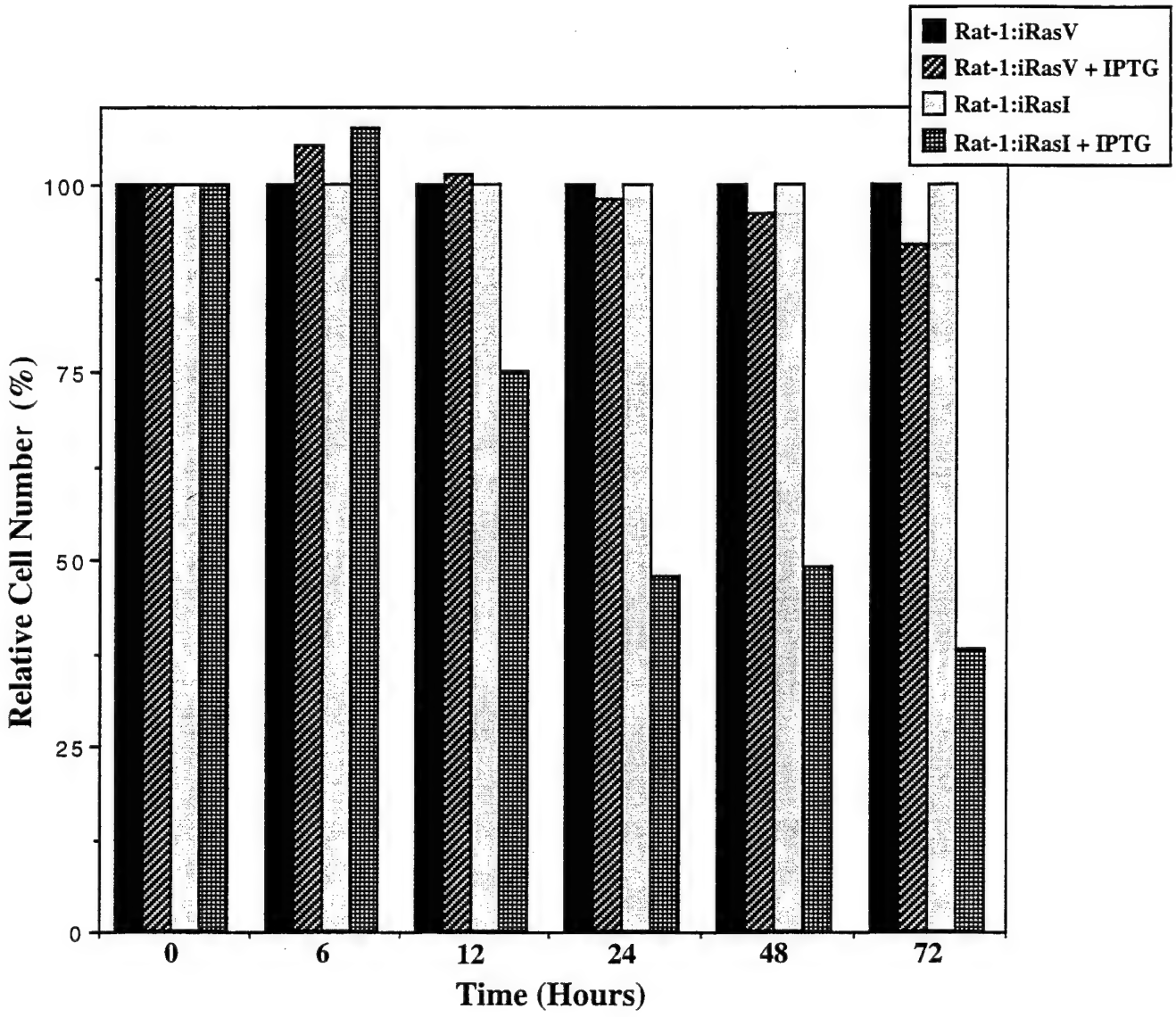

Rat-1:iRasV
Rat-1:iRasV + IPTG
Rat-1:iRasI
Rat-1:iRasI + IPTG
Relative Cell Number (%)
100
75
50
25
0
0
6
12
24
48
72
Time (Hours)

Figure 3B Mayo et al.

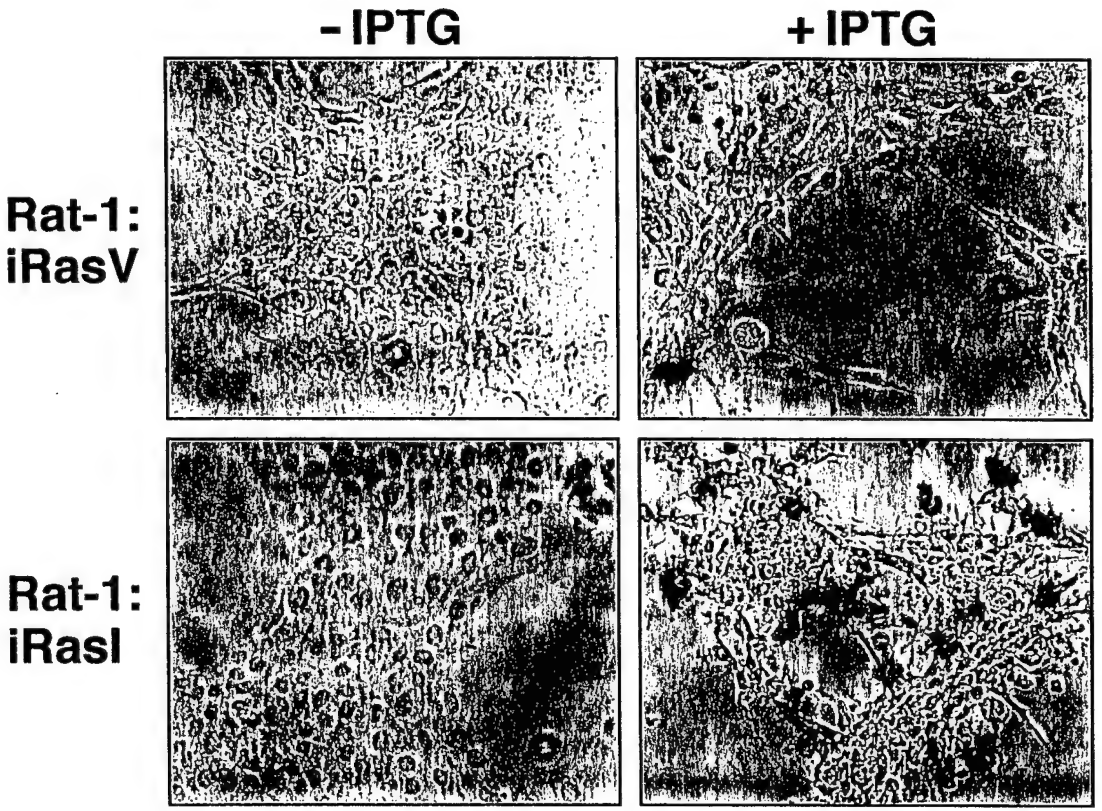


Figure 4A

A.

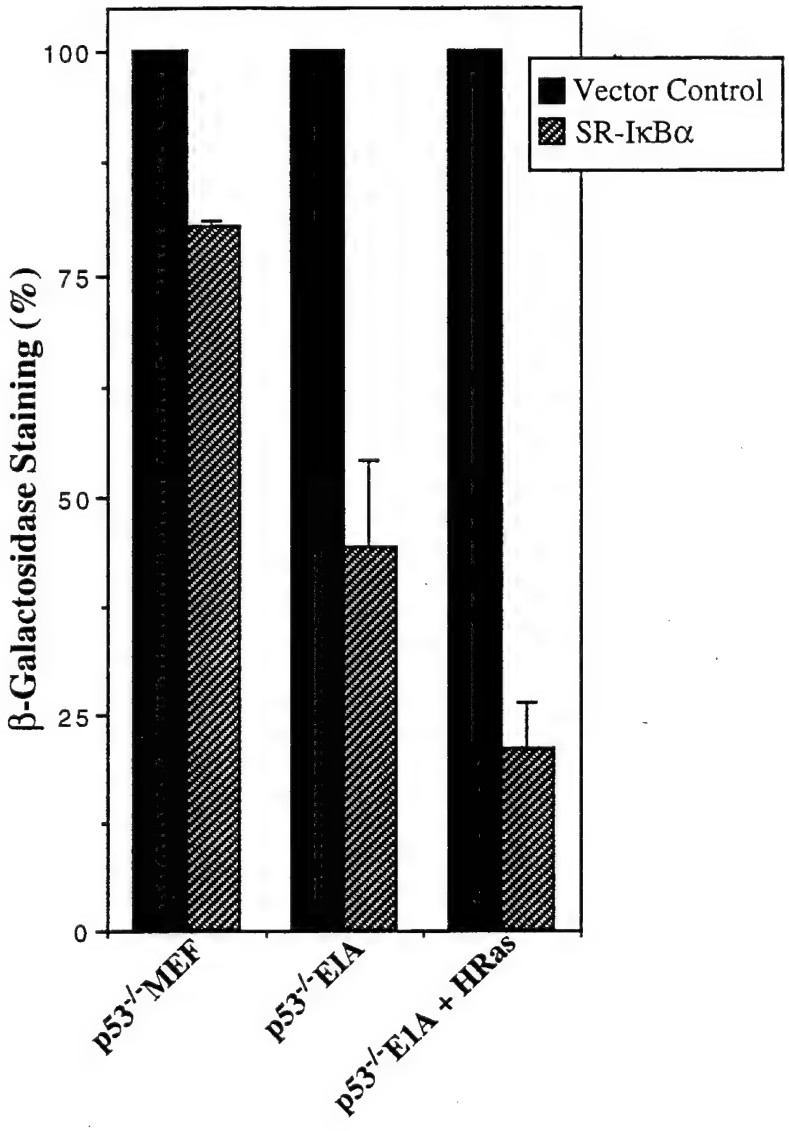
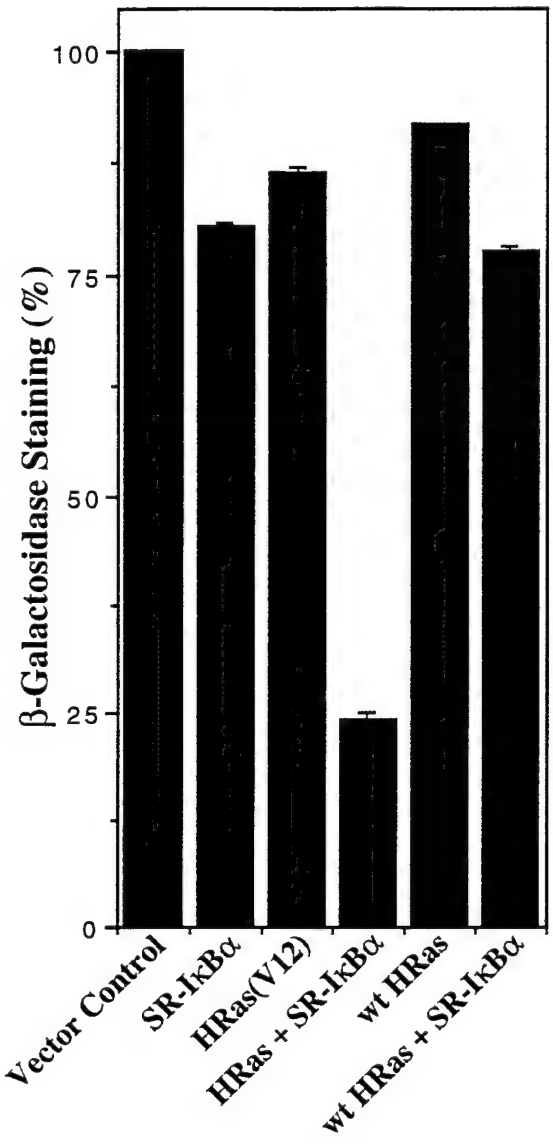

100
75
50
25
0
β-Galactosidase Staining (%)
Vector Control
SR-IκBα
p53-/-MEF
p53-/-E1A
p53-/-E1A + HRas

Figure 4B

B.


β-Galactosidase Staining (%)
100
75
50
25
0
Vector Control
SR-IκBα
HRas(V12)
HRas + SR-IκBα
wt HRas
wt HRas + SR-IκBα

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(J. Biol. Chem.)

Oncogenic H-Ras-Induced Signaling Activates NF-κB Transcriptional Activity Which is Required for Cellular Transformation

Timothy S. Finco*@, John K. Westwick*+, Jacqueline L. Norris*,
Amer A. Beg#, Channing J. Der*@ and Albert S. Baldwin, Jr.*@^

*Lineberger Comprehensive Cancer Center, @Curriculum in Genetics and Molecular Biology, +Department of Pharmacology, ^Department of Biology, University of North Carolina, Chapel Hill, NC 27599-7295. #Department of Biological Sciences, Columbia University, New York, NY 10027.

RUNNING TITLE: Requirement of NF-κB for Ras-mediated transformation

To whom correspondence should be addressed:

Albert S. Baldwin, Jr.
Lineberger Comprehensive Cancer Center
CB# 7295
University of North Carolina School of Medicine
Chapel Hill, NC 27599

Phone: (919) 966-3652
FAX: (919) 966-0444

Present Addresses: T.S.F., Howard Hughes Medical Institute, University of California, San Francisco; J.K.W., Signal Pharmaceuticals, San Diego, CA.

Summary:

Ras proteins function in stimulating cell proliferation and differentiation through the activation of Raf-dependent and Raf-independent signal transduction pathways and the subsequent activation of specific transcription factors. For example, Ets and AP-1 family proteins are activated by Ras-induced pathways and are required for oncogenic Ras to induce cellular transformation and tumorigenesis. The transcription factor NF-κB has been widely studied as a regulator of genes involved in immune and inflammatory responses. A variety of stimuli activate NF-κB through the induced phosphorylation and degradation of the inhibitor IκB followed by nuclear translocation of NF-κB. We show here that oncogenic forms of H-Ras activate NF-κB, not through induced nuclear translocation, but rather through the activation of the transcriptional function of the NF-κB RelA/p65 subunit. Importantly, RelA/p65 -/- cells are inefficient in the activation of κB-dependent gene expression in response to oncogenic Ras expression. Furthermore, IκBα expression blocks focus formation in NIH3T3 cells induced by oncogenic Ras. These results demonstrate that NF-κB is a critical downstream mediator of H-Ras signaling and oncogenic potential.

INTRODUCTION:

Members of the ras family of GTP binding proteins serve as essential mediators in the ability of a variety of extracellular stimuli to regulate cellular proliferation and differentiation (1,2). Oncogenic mutations in *ras* alleles, which occur in approximately 30% of human cancers, lead to chronic GTP binding which initiates the activation of signal transduction cascades. In this regard, Ras is known to stimulate both the RAF/MEK/ERK pathway as well as the MEKK/SEK/JNK pathway (3-7). Activation of these and other protein kinase cascades (8-10) is critical for the ability of Ras to exert both its normal and oncogenic functions. The ultimate targets of the Ras-induced signal transduction pathways are transcription factors (see 4), which regulate the expression of genes involved in proliferation and oncogenesis. Two transcription factors, Ets and c-Jun, have been shown to be essential for Ras-induced gene expression and for Ras-mediated cell transformation in vitro and tumorigenesis (11,12). In these cases, Ras-induced signaling pathways activate the transcriptional function of both Ets and c-Jun via induced phosphorylation of their transcriptional activation domains (13, and reviewed in 4).

The NF-κB family of proteins has been studied largely for the ability of these transcription factors to regulate a variety of genes involved in immune and inflammatory responses (reviewed in 14). The activation of these genes in response to inflammatory cytokines, T cell activation signals, LPS, etc. involves the targeted phosphorylation and degradation of the NF-κB inhibitor IκB, allowing nuclear translocation of NF-κB (reviewed in 14). Gene knockout studies (see 14) demonstrate that NF-κB/Rel proteins play important roles in normal immunological responses. Additionally, growing evidence indicates that NF-κB may play an important role in controlling cellular proliferation. For example, the c-*myc* proto-oncogene has been shown to be transcriptionally regulated by NF-κB (15). Additionally, numerous viral transforming proteins activate NF-κB (reviewed in 14) and antisense inhibition of IκBα leads to cellular transformation of NIH 3T3 cells (16). Furthermore, members of the NF-κB and IκB

families are associated with chromosomal translocations found in certain lymphomas (for example, see 17) and the founding member of the NF-κB family, c-Rel, is the cellular homologue of the transforming gene of avian reticuloendotheliosis virus.

We and others previously demonstrated that transient transfection of oncogenic forms of H-Ras or of Raf-1 leads to the activation of reporter gene expression controlled by multiple NF-κB sites (18, 19). In order to extend our studies, we analyzed NIH 3T3 cells transformed by oncogenic H-Ras or by oncogenic Raf-1. Consistent with the previous co-transfection studies, κB-dependent gene expression was elevated significantly in both the Ras- and the Raf-transformed cells as compared to the parental 3T3 cells. Interestingly, increased NF-κB binding activity was not detected in the Ras- or Raf-transformed cells. However, the activity of the transcriptional activation domain of the NF-κB RelA/p65 subunit was significantly increased in these cells. p65-/- fibroblasts exhibited a reduced κB-dependent transcription response to either oncogenic Ras or Raf, but retained their ability to activate the p65/RelA transcriptional activation domain. Finally, oncogenic Ras focus-forming activity was blocked by IκBα expression. These data indicate that NF-κB is a downstream target for Ras-activated signal transduction pathways, that oncogenic Ras stimulates the transcriptional function of the p65/RelA subunit of NF-κB, and that NF-κB is required for cellular transformation mediated by oncogenic *ras* alleles.

EXPERIMENTAL PROCEDURES:

Cells and transfections:

NIH 3T3 cells, the H-Ras and Raf-1 transformed counterparts, and the p65+/- and p65-/- mouse embryo fibroblasts were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, penicillin, and streptomycin. DNA transfections were performed by the calcium phosphate precipitation method as previously described (18). The plasmid pGEM or salmon sperm DNA was used to equalize the amount of DNA transfected in each experiment to 15 μg. CAT analysis and luciferase assays were performed as previously reported (18,20). In all cases, one unit of relative activity represents the CAT or luciferase activity obtained after transfection of the reporter gene alone. All experiments were performed at least three times with similar results.

Plasmids:

The following plasmids have been described previously: activated Raf (RafBXB) and activated Ras (v-H-Ras) expression vectors (18), the IκBα expression vector (21), the super-repressor IκBα expression vector (22), the expression vector encoding the Gal4 DNA binding domain fused to the C-terminal domain of p65/RelA (Gal4p65aa519-551, 23), the reporters 3X-κB-CAT and 3X-mutκB-CAT (18), the HIV LTR-CAT and HIV-ΔκB-CAT reporters (18), 5X-Gal4-CAT (24), and DHFR-CAT (25).

Extracts and Gel Mobility Shift Assays:

Nuclear and cytoplasmic extracts were prepared as previously described (26). For double sucrose pad purification, washed nuclei were resuspended in lysis buffer lacking NP-40 and layered on a sucrose pad (30% sucrose, 60 mM KCl, 15 mM NaCl, 15 mM Hepes, 2 mM EDTA,

0.75 mM spermidine, 0.15 spermine, and 1 mM DTT) and centrifuged for 15 min at 3,000 rpm in an HB4 rotor. The sucrose pad was then removed, the nuclei resuspended and the process repeated. Nuclear extracts were then prepared from the sucrose pad-purified nuclei. Nuclear extracts were centrifuged for 10 min at 14,000 rpm to remove particulate matter and the subsequent supernatant (nuclear extract) was transferred to new tubes and samples were stored at -70°C until needed. Gel mobility shift assays (EMSAs) were performed as previously described (26).

Focus Formation Assays:

NIH 3T3 cells were transfected by calcium phosphate coprecipitation essentially as described (27). For each 60 mm plate, 10 ng of Ras expression vector pZip-*ras*H(61L) was transfected, along with the indicated quantity of IκBα expression vector or empty vector. In all cases, the expression vector was normalized with the cognate empty expression vectors. Cells were fed every two days and the appearance of foci of transformed cells were counted 14 days after transfection. Four plates per condition were transfected, and graphs represent the mean +/- SEM of these counts. Data are representative of four independent experiments performed in quadruplicate.

RESULTS:

Oncogenic Ras- or Raf-transformed cells exhibit increased κB-dependent transcription without increased nuclear accumulation of NF-κB. Previous transient cotransfection experiments indicated that expression of either oncogenic Ras or oncogenic Raf led to a significant activation of expression of a κB-dependent reporter (18). Consistent with the previous cotransfection data, the activity of a κB-dependent reporter was significantly elevated in both Ras- and Raf- transformed cells, but not in the parental NIH3T3cells (Fig. 1A). A reporter mutated in the NF-κB sites did not exhibit this enhanced activity and expression of the NF-κB inhibitor IκBα blocked the Ras- and Raf-induced activation of κB-dependent reporter activity, indicating that NF-κB regulates the transcription response. A similar result was obtained with the NF-κB dependent HIV-LTR reporter (Fig. 1B). Additionally, expression of the non-Ras responsive DHFR-CAT reporter was approximately equivalent in each of the three cell types (Fig. 1C) showing that the differential responses observed in the transformed cells were not due to differential uptake of plasmids.

In order to determine if oncogenic Ras as well as oncogenic Raf activated nuclear accumulation of NF-κB, gel mobility shift assays (EMSAs) were performed with nuclear extracts from parental NIH3T3 cells, oncogenic Ras-transformed NIH3T3 cells, or Raf-transformed NIH3T3 cells. To demonstrate that binding activity was exclusively nuclear, extracts were prepared from double sucrose pad purified nuclei. Immunoblotting for NF-κB1/p105 (which is cytoplasmic) indicated that there was no cytoplasmic contamination in the nuclear preparations (data not shown). As shown in Fig. 2, NF-κB was detected in the nuclei of each of the different cells at similar levels. Antibody "supershift" experiments showed that this binding activity is authentic, p65/RelA-containing NF-κB (data not shown). These results were surprising and indicated that the activation of κB-dependent transcription observed in the transfection

experiments shown in Figure 1 were not controlled by the induced nuclear accumulation of NF-κB but suggested that this response was mediated by the relatively low levels of constitutively nuclear NF-κB in NIH3T3 fibroblasts. It should be noted that transient transfection of oncogenic Ras into 3T3 cells or the induction of oncogenic Ras in Rat-1 cells leads to an approximate 3-fold increase in nuclear NF-κB (data not shown), however established Ras-transformed cells do not exhibit this property. These experiments indicate that oncogenic Ras or Raf can activate κB-dependent transcription without enhancing nuclear levels of NF-κB.

Oncogenic Forms of Ras and Raf Activate the Transcriptional Function of NF-κB p65/RelA. To explain the activation of κB-dependent trancription by oncogenic forms of Ras or Raf without an induction of NF-κB nuclear translocation, we asked whether the transcriptional activation function of NF-κB was stimulated in the transformed cells. A plasmid (Gal4p65) encoding a fusion of the C-terminal (TA1) transactivation domain of RelA (23) with the DNA binding domain of the yeast transcription factor Gal4 was transfected into parental NIH3T3 cells, or Ras- or Raf-transformed cells, along with a luciferase reporter containing upstream Gal4 binding sites. Luciferase activity driven by Gal4p65 or Gal4 was compared in the three cell types. The results indicate that the Gal4p65 construct is strongly active in the Ras- and the Raf-transformed cells but only weakly active in the untransformed 3T3 cells (Figure 3A). EMSA experiments indicated that there was not an increase in the DNA binding activity of the Gal4-p65 protein in the Ras- and Raf- transformed cells (data not shown). These results demonstrate that oncogenic Ras or Raf activates a signal transduction pathway that stimulates p65/RelA transcriptional activation function controlled by the TA1 transcriptional activation domain.

The results described above suggested that the RelA subunit of NF-κB may function as a critical downstream transcriptional effector for the Ras oncoprotein. In order to test this

hypothesis, we utilized immortalized RelA+/- and RelA-/- embryonic fibroblasts (28) for transfection and gene expression studies. Oncogenic Ras was ineffective at activating κB-dependent gene expression in the p65-/- cells (approximately a 2-fold activation) whereas effective Ras-activation of κB-dependent gene expression (approximately 7-fold) was observed in the RelA+/- cells, as expected (Fig. 3B). To show that the Ras-responsive signal transduction pathway was still operative in the RelA-/- cells, the Gal4p65 construct was cotransfected with either activated H-Ras or activated Raf-1. Ras activated the Gal4p65 construct as effectively in RelA+/- cells as in RelA-/- cells. These results demonstrate that the RelA/p65 subunit of NF-κB is required for oncogenic Ras to effectively activate gene expression driven by consensus NF-κB binding sites (also see discussion).

NF-κB is required for Ras-mediated cellular transformation. In order to determine whether NF-κB is required for cellular transformation controlled by oncogenic H-Ras, we determined whether the inhibition of NF-κB would affect the ability of Ras to cause formation of transformed foci in cultured NIH3T3 cells. In order to specifically inhibit NF-κB activity, we used an expression vector encoding IκBα, which can enter the nucleus and relocate NF-κB to the cytoplasm (29). Transfection of pZIP-*ras*(61L) together with the empty CMV vector yielded an average of approximately 160 foci per plate (Figure 4). Co-expression of oncogenic Ras with wild-type IκBα blocked focus formation activity by greater than 50%. Co-expression with a super-repressor form of IκBα (mutated in serines 32 and 36) that is unable to be inducibly phosphorylated or degraded in response to stimuli (see 22) blocked focus formation by approximately 70 to 75% (Figure 4). Expression of IκBα did not block expression of the promoter driving Ras expression or Ras protein expression (data not shown). Interestingly, IκBα was unable to block the ability of activated Rho (Rho63L) to induce focus formation. In these experiments, activated Rho yielded approximately 20 foci per plate and IκBα expression

did not reduce this number of foci (data not shown). Immortalized p65 -/- cells exhibited an NF-κB binding activity which prevented the use of these cells in transformation studies (see discussion). In summary, the data indicate that oncogenic Ras activates gene expression via the functional activation of the p65/RelA transcriptional activation domain and that NF-κB is required for Ras-mediated cellular transformation.

DISCUSSION:

The data presented here indicate that oncogenic *ras* alleles activate NF-κB dependent transcription, not through the induced nuclear translocation of NF-κB, but rather through the stimulation of the transcriptional activation function of NF-κB via the targeting of the RelA/p65 subunit. Furthermore, the data indicate that NF-κB is required for Ras to initiate efficient cellular transformation and that NF-κB plays a role in mediating certain essential aspects of cellular transformation. Thus, NF-κB joins Ets family members (13) and c-jun (4,12) as downstream targets of oncogenic Ras that are required for Ras-mediated cellular transformation.

How does Ras activate NF-κB functional activity? Our data strongly indicate that the transcriptional activation function of the RelA/p65 NF-kB is potentiated in both Ras- as well as as Raf-transformed cells and at least two mechanisms exist to explain this phenomenon. First, a Ras-initiated signal transduction pathway may target the p65 transcriptional activation domain for phosphorylation which may allow enhanced interactions with a transcriptional co-activator or with basal transcriptional machinery. Such a mechanism appears to be operative for both Ets-1 and 2 and for c-jun (4,13). A second mechanism may be that a transcriptional co-activator is modified such that it interacts functionally with the p65 TAD. Recently, Montminy and colleagues (30) showed that oncogenic Ras targets the CBP/p300 transcriptional activator to stimulate transcription directed by c-Jun but inhibits transcription directed by CREB. This regulation was controlled by pp90RSK but presumably is also controlled by the inducible phosphorylation of the transcription factors. Recently, it was shown that CBP/p300 functionally interacts with the p65/RelA transcritpional activation domain (31,32). Present experiments are aimed at determining whether these observations are relevant to the ability of Ras to stimulate the transcriptional activation function of NF-κB. Also of importance is the identification of the signal transduction pathway that is initiated by Ras to stimulate NF-κB transciptional activity.

Since both oncogenic Ras as well as oncogenic Raf stimulate κB-dependent activity, it may be assumed that the relevant pathway is downstream of Raf and is, therefore, the MEK/ERK pathway. However, inhibitors of this pathway did not block the ability of Ras to activate κB-dependent transcription and dominant negative forms of kinases in the SEK/JNK pathway were able to block this response (J. Norris and A.B., unpublished). Thus the ability of Raf to activate κB-dependent gene expression in a MEK/ERK-independent pathway may be explained by the recent observation that Raf stimulates JNK activity via an autocrine mechanism (33).

Our data show that cells that are null for p65/RelA exhibit reduced κB-dependent transcription in response to oncogenic Ras expression (Fig. 3). We have found that immortalized p65-/- cells exhibit constitutive nuclear expression of a form of NF-κB that appears to contain c-Rel. Since c-Rel can potentially regulate transcription through non-consensus κB-sites (see14) and since we observe a weak transcriptional reponse driven by the class I MHC NF-κB binding site, it is likely that the immortalized p65-/- null cells are not functionally NF-κB null. Consistent with this hypothesis, we have found that immortalized p65-/- fibroblasts that express oncogenic Ras can form tumors in nude mice. For this reason, we used IκBα expression (which can block both p65 and c-Rel) to assess a role for NF-κB in cellular transformation controlled by oncogenic Ras. As shown in Figure 4, IκBα effectively suppressed the formation of transformed foci induced by oncogenic Ras. Future studies will determine if c-Rel is activated by oncogenic Ras in a manner similar to that observed for p65/RelA.

Prior studies have shown that the major regulatory mechanism involved in regulating κB-dependent transcription is induced nuclear translocation (see 14). Our data indicate that significant κB-dependent transcription can be realized without enhancing the constitutive, low nuclear levels of NF-κB. This suggests that under some circumstances the functional activity of NF-κB can be separated from induction of nuclear translocation. Consistent with this concept are the recent observations that the tyrosine kinase inhibitor genistein blocks the ability of NF-

κB to stimulate transcription of an NF-κB dependent reporter but is not able to block nuclear translocation of NF-κB (34) and that PMA can activate the TA2 transcriptional activation domain of RelA/p65 (23).

Evidence that NF-κB is required for Ras-mediated cellular transformation is consistent with several observations indicating a role for NF-κB in controlling cell growth. First, it has been shown that NF-κB can regulate c-*myc* gene expression. Second, antisense studies indicate that NF-κB can control oncogenesis. These experiments utilized antisense to p65 to block oncogene-controlled transformation (35, 36) and antisense to IκBα to induce transformation of NIH 3T3 cells (16). Additionally, other oncogenes such as Her2/NEU are known to activate NF-κB (37). Thus, the activation of NF-κB may be common to a number of oncogenes, particularly those that utilize Ras-controlled signaling pathways. Additionally, we have been able to show NF-κB activation is required to block a Ras-induced apoptotic response (M. Mayo and A.B., submitted). This result is consistent with recent data (22,38-40) that NF-κB activation can block the induction of apoptosis. Further experiments are required to establish the exact role that NF-κB plays in controlling Ras-mediated oncogenesis. Since NF-κB inhibitors exist, it will be important to determine if the inhibition of NF-κB will serve as a primary line of therapy for Ras-controlled tumors.

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FIGURE LEGENDS:

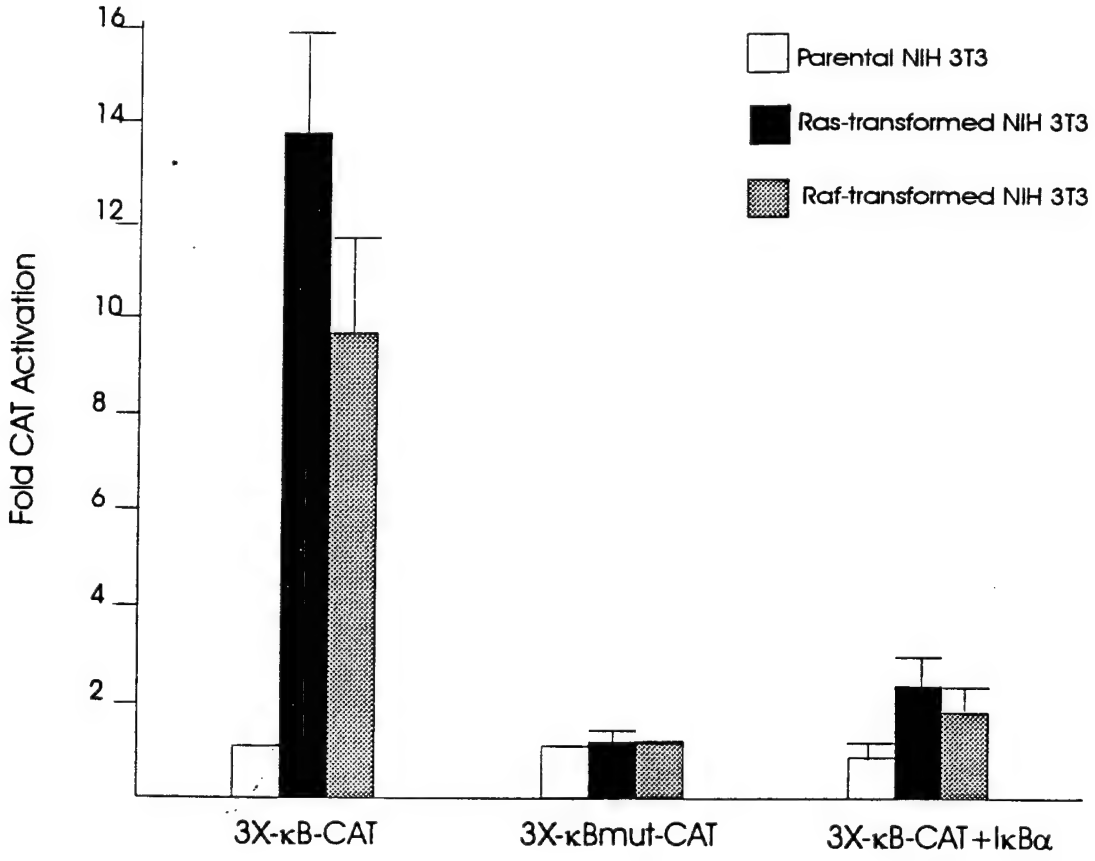
Figure 1. κB-dependent gene expression is increased in Ras- as well as Raf-transformed cells. (**A**) Either the κB-dependent CAT reporter (3X-κB-CAT) or its mutant version (3X-κBmut-CAT) were transfected into Ras-transformed NIH3T3 cells, Raf-transformed NIH3T3 cells or parental NIH3T3 cells either alone or with the CMV-IκBα expression vector. CAT activity was measured as described in Experimental Procedures. (**B**) Identical experiment as shown in A except that the HIV-LTR-CAT vector or the version mutated in the NF-kB sites (HIV-LTRΔκB-CAT) were used. (**C**) DHFR-CAT was transfected into the 3 different cells and CAT assays performed. Data are presented as mean +/- s.d.

Figure 2. NF-κB binding activity is not increased in Ras- or Raf-transformed NIH3T3 cells. Gel mobility shift assays were performed on extracts of double sucrose pad purified nuclei from parental NIH3T3 cells, or the Ras- and Raf-transformed counterparts as described in the Experimental Procedures. NF-κB is shown by the arrow.

Figure 3. The p65/RelA subunit of NF-κB is functionally activated by Ras and is required for Ras to efficiently activate κB-dependent gene expression. (**A**) Either the vector (Gal4p65) encoding a fusion protein between the DNA binding of Gal4 and the TA1 transcriptional activation domain of p65/RelA or the Gal4 vector was transfected into NIH3T3 cells or the Ras- or Raf-transformed counterparts of these cells. CAT activity was determined as described in Experimental Procedures. (**B**) SV40 large T (Tag) immortalized embryonic fibroblasts isolated from p65-/- or p65+/- mice were transfected with the κB-dependent CAT reporter alone with oncogenic Ras or with the Gal4p65 vector alone or with oncogenic Ras. CAT activity was measured as described and is presented as mean +/- s.d.

Figure 4. IκBα blocks focus formation induced by oncogenic Ras. NIH3T3 cells were transfected with the oncogenic Ras expression vector (plus empty CMV vector) or with the empty vector Ras expression vector. Alternatively, the Ras expression vector was co-transfected with a vector encoding the wild-type form of IκBα or a vector encoding the modified, super-repressor form of IκBα (IκBAA) as described in the Experimental Procedures. Results are presented as foci per plate and are the mean +/- s.d.

A.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Raf-transformed NIH 3T3
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3X-κBmut-CAT
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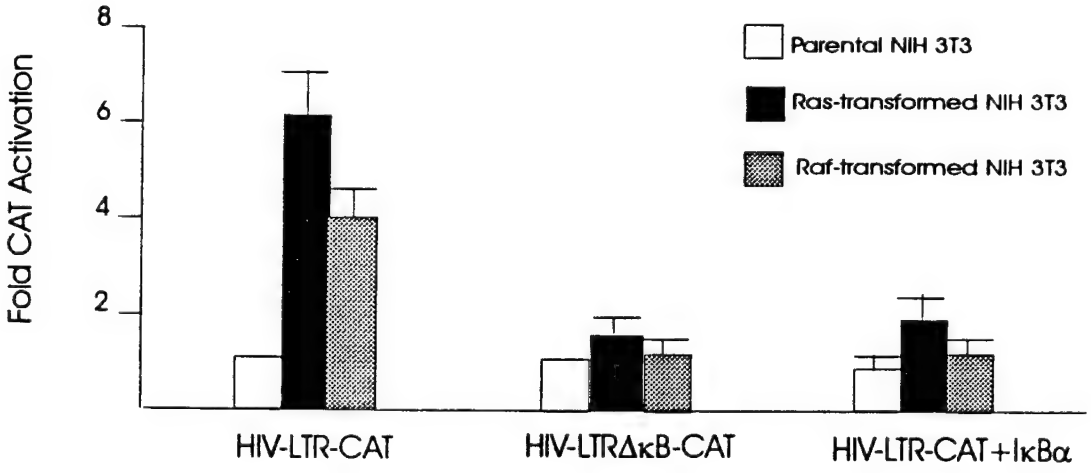
B.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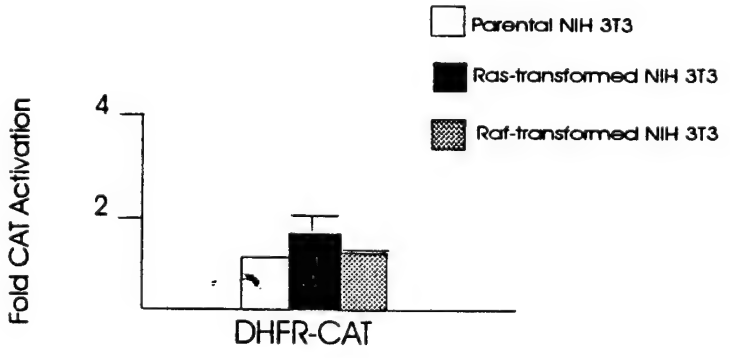
C.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parental
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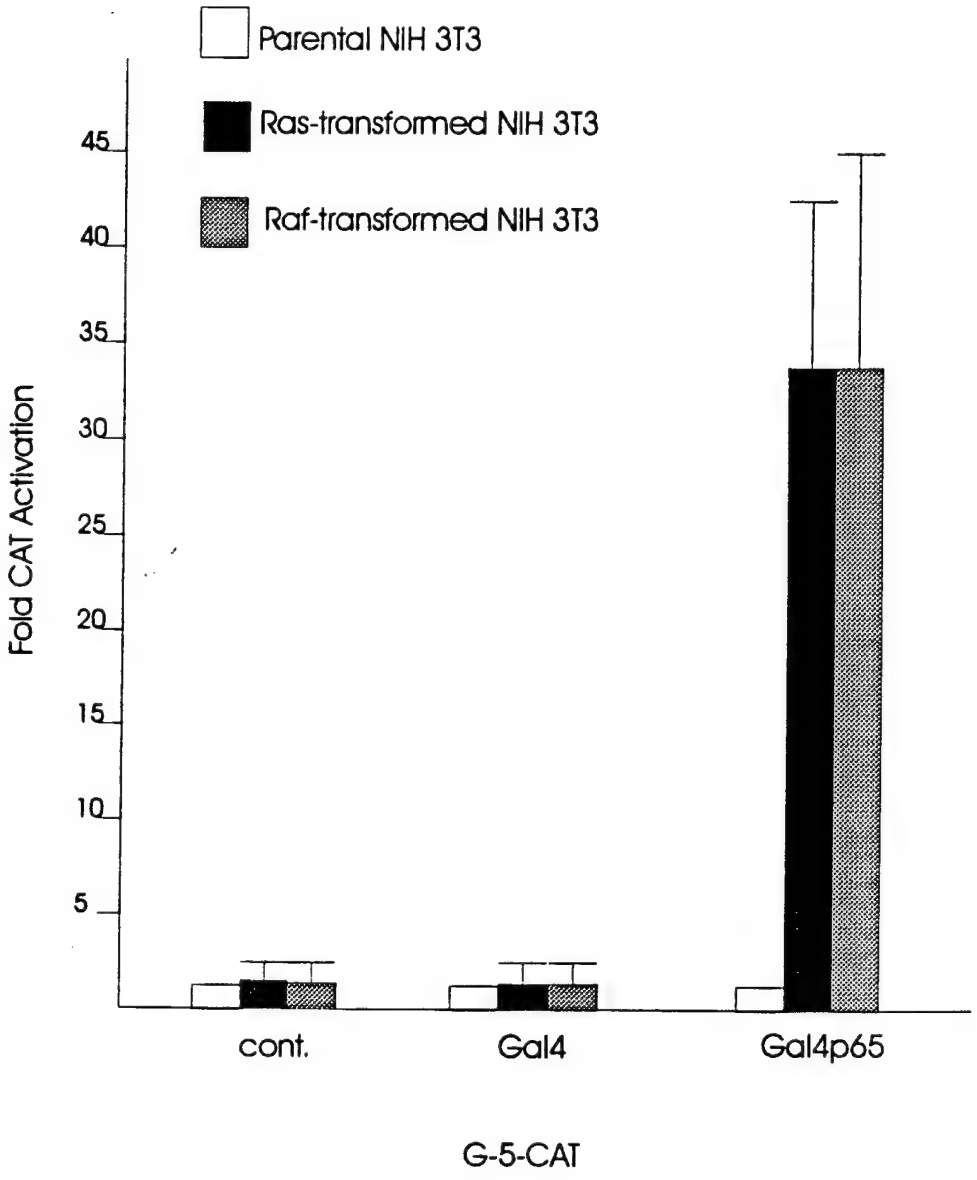
Raf
NF-κB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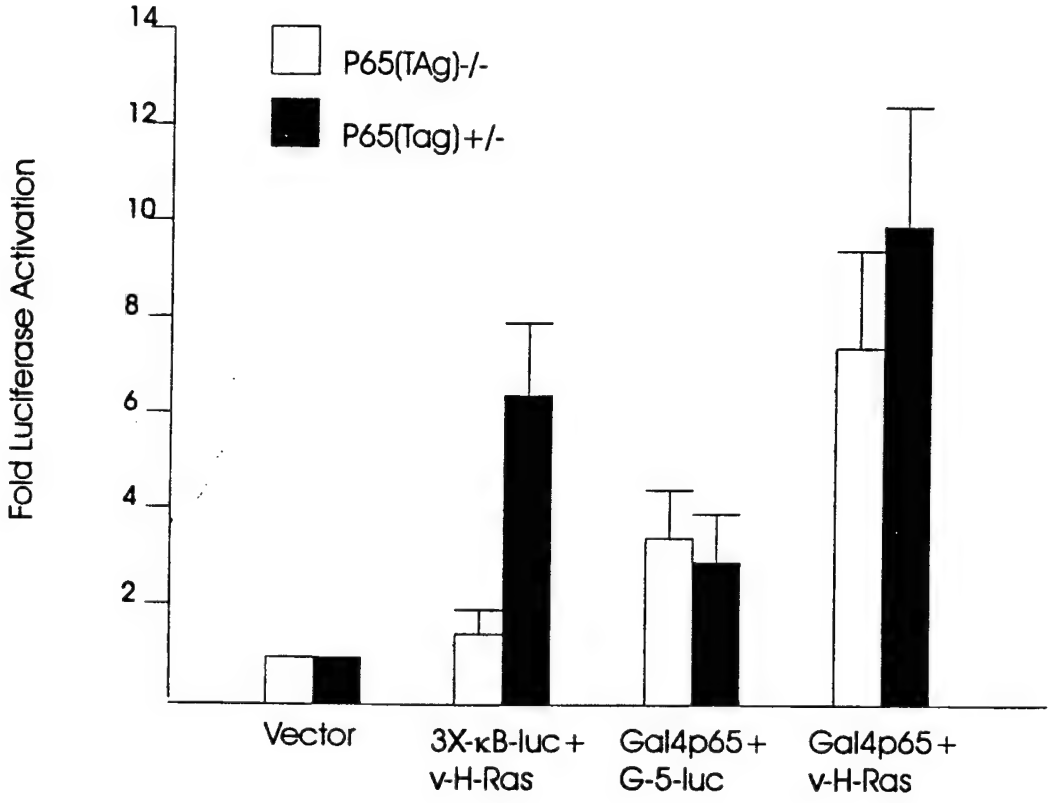
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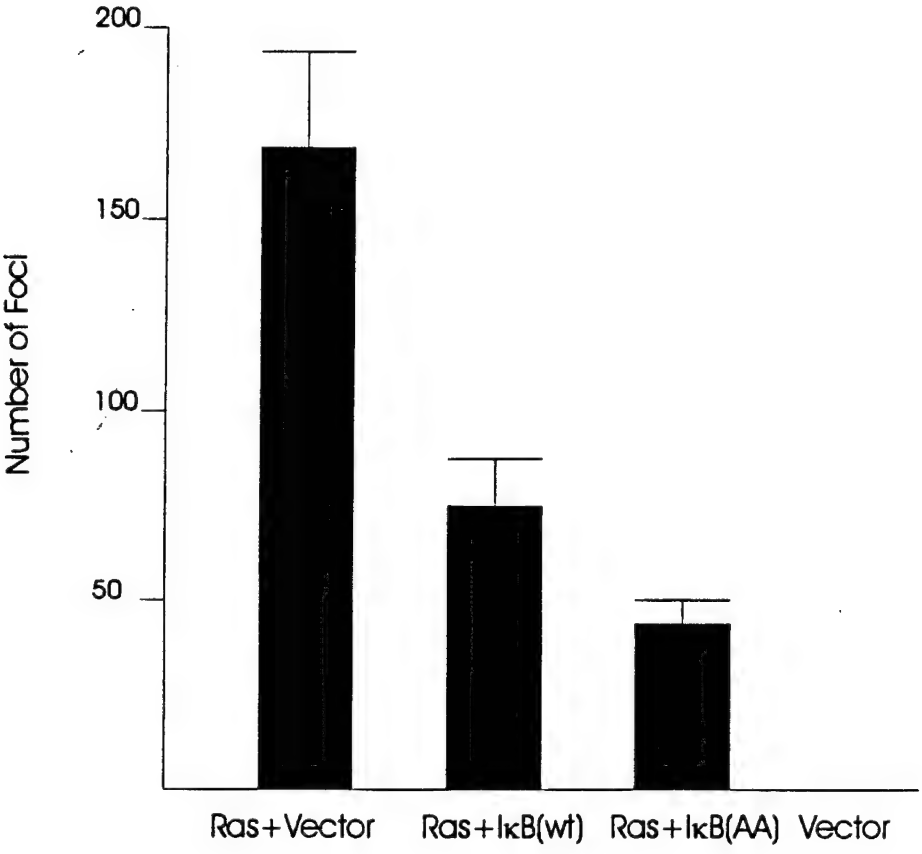
200
150
100
50
Number of Foci
Ras+Vector
Ras+IκB(wt)
Ras+IκB(AA)
Vector

In Press
(Cancer Rer.)

Potentiation of Camptothecin-induced Apoptosis by Inhibition of NF-κB[1]

Cun-Yu Wang, Marty W. Mayo, James C. Cusack, and Albert S. Baldwin[2]

Lineberger Comprehensive Cancer Center [C. Y. W., M. W. M., J. C., A. S. B.], Curriculum in Genetics and Molecular biology [C. Y. W., A. S. B.], Department of Endodontics of the School of Dentistry [C. Y. W.], Department of Surgery [J. C.], Department of Biology [A. S. B.], University of North Carolina at Chapel Hill, North Carolina 27599-7295

Running Title: Control of Camptothecin-Induced Apoptosis

Keywords: Apoptosis, NF-κB, Caspases, Topoisomerase I inhibitor, CPT-11/SN38

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[2] To whom correspondence should be addressed, at Lineberger Comprehensive Cancer Center, Campus Box 7295, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, Phone: (919)966-3652, Fax: (919)9663015

[3] The **abbreviations** used are: Topo I, topoisomerase I; NF-kB, Nuclear factor Kappa-B, CPT, Campothecin, ICE, interleukin-1-converting enzyme, TUNEL, Deoxynucleotidyl transferase-mediated dUTP nick end labeling, EMSA, Electrophoretic mobility shift assay,

Abstract

The transcription factor nuclear factor kappa B (NF-kB) plays an important role in inflammation and immunity, and can be activated by multiple inducers such as tumor necrosis factor and chemotheraputic drugs. In a human fibrosarcoma cell line, we found that Topoisomerase I inhibitors Camptothecin (CPT) and derivative SN38 activate the nuclear translocation of NF-kB in time- and concentration-dependent manner. Furthermore, we demonstrated that the cell increased sensitivity to CPT-induced apoptosis by inhibition of NF-kB signaling, and enhancement of CPT-induced apoptosis was dependent on caspases. It suggest that NF-κB-mediated genes may inhibit caspases. Our results identified a new mechanism of cell sensitivity to CPT and its derivatives, and provided a molecular basis for altering intrinsic cell susceptibility to the treatment of CPT and its derivatives.

Human Topo I is a nuclear enzyme that plays an important role in DNA replication and transcription (1). The level of Topo I is found to be elevated in several types of malignant tumor such as colorectal cancer, and it has become an attractive target for chemotheraputic compounds such as CPT and its derivatives (2-4). CPT is an alkaloid agent as an antitumor drug originally isolated from the oriental plant

Camptotheca acuminata. CPT and its derivatives are considered as the inducer of DNA damage through trapping Topo I-cleavable complexes and inhibiting the religation of the topo I reaction. Based on studies in both in cultured cells and in cell-free extracts, the fork collision model has been proposed to explain the sensitivity of S phase. CPT-induced Topo I breaks are by themselves not sufficient for cell death. Collision between an ongoing replication fork and a topo I-CPT-DNA ternary complex, the replication fork is irreversibly arrested and a double-strand break generated. These double-strand breaks are stabilized and induce a series of unknown events to lead cell death (2-5). CPT and its analogue have been reported to kill tumor cell through apoptotic mechanism that internucleosomal DNA fragmentation is a common hallmark of apoptosis (6-10). CPT11, one of analogues of CPT has been reported that it has broad antitumor function against colorectal, small cell and non-small cell lung, cervical, ovarian, and breast cancer; soft tissue sarcomas; osteosarcoma, mesotheliomas, and leukemias. It is one of the most promising anticancer drugs presently in clinical trials (11).

NF-kB, originally identified and named for its role in regulation of immunoglobulin kappa chain gene expression in B cells, is a heterodimer of a 50-kDa subunit (p50) and a 65-kDa subunit (p65; RelA). Presynthesized NF-kB is sequestered in cytoplasm by a group of inhibitory proteins known as IkB. NF-kB is activated by various stimuli such as tumor necrosis factor, IL-1, LPS, growth factor, viral infection and radiation (12-13). Recently, we established a human fibrosarcoma cell line (HT1080I) expressing a super-repressor form of the NF-kB inhibitor IkBa. The super-repressor IkBα contains serine-to-alanine mutation at residues 32 and 36, which is resistant to signal-induced phosphorylation and subsequent proteasome-mediated degration. In this model cell system, we found the activation of the transcription factor nuclear factor-kappa B (NF-kB) by tumor necrosis factor and a chemotherapeutic compound daunorubicin provided the survival function (14). In the present study, we demonstrated that CPT and its derivative SN38 strongly activate NF-kB. Furthermore, we observed an enhancement of CPT-induced apoptosis through blocking the NF-kB signaling in HT1080I cell.

Materials and Methods

Cell Cultures. HT1080I and a control cell line (HT1080V) were cultured in EMEM medium supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, 100 mg/ml streptomycin, and 200 mg/ml hygromycin. CPT was purchased from Sigma. CPT-11 (irinotecan) and its active metabolite, SN-38, were kindly provided by Upjorn Company. Tripeptide ICE inhibitor, z-VAD-fmk (z-Val-Ala-Asp-fluoromethyl ketone) was purchased from Enzyme system (15-16).

Nuclear extracts and EMSA. Cell was treated with the indicated concentration of CPT or SN38. Nuclear extracts were prepared as described (14). 5 mg nuclear extracts of cells were preincubated with 1 mg of poly(dI-dc) in binding buffer (10 mM Tris, 50 mM NaCl, 20 % glycerol, 1 mM DTT, 0.5 mM EDTA) for 10 min at room temperature. Approximately 20,000 cpm of ^{32}P-labelled DNA probe containing the class I MHC NF-kB site was then added and allowed bind for 15 min. The complexes were separated on a 5 % polyacrylamide gel and autoradiograghed (14). Anti-p65 and p50 antibodies for supershift were purchased from the Santa Cruz.

Western blot analysis. Whole cell extracts were subjected to sodium dodecyl sulfate 10% polyacrylamide gel electrophoresis and transferred to nitrocellulose by electroblotting. The protein was probed by primary antibody and visualized by using an ECL kit (Amersham) according to the manufacturer's instruction. Polyclonal antibodies against human Bcl-2, Bcl-x_l were purchased from Santa Cruz.

TUNEL assays, cell cytotoxicity assay and cell death ELISA. For TUNEL staining, the cells were either untreated or treat with 1 mg/ml SN38 for 8 hr and fixed with 4 % paraformaldehyde 30 min at room temperature. The cell were washed in PBS and incubated in permeabilisation solution (0.1% Triton X-100, 0.1% sodium citrate) for 2 min on ice. The cells were labeled with TUNEL reaction mixture. The incorporated fluorecein signal was converted by anti-fluorescein antibody conjugated with alkaline

phosphatase. The color was developed by substrate consisting of NBT and BCIP. For cell cytotoxicity assays, the cells were treated with CPT or SN38 for 24 hr. The surviving cells were stained by 0.05% crystal violet in 20% EtOH. The crystal violet density was quantified in 590 nm length by kinetic microplate reader (14). For cell death ELISA, the cells were preincubated for 10 mM Z-VAD for 30 min and treated by SN38 for 16 hr. 50 ul of cell culture supernatant was analyzed by a cell death detection ELISA kit according to manufacturer's instruction (Boehringer Mannheim)

Results

CPT and SN38 activate NF-kB. To determine whether CPT activates NF-kB, nuclear extracts from the CPT or SN38-treated cells were analyzed by EMSA using the major histocompatibility complex class I NF-kB DNA binding site. As shown in Fig. 1A (lane 1-4), the activation of NF-kB by CPT was at concentration as low as 0.1uM and in the dose-dependent manner. The nuclear translocation of NF-kB is a classical heterodimer of a 50-kDa subunit (p50) and a 65-kDa subunit (p65, RelA) confirmed by supershift with p50 and p65 antibodies (unpublished data). Interestingly, NF-kB activation reached a maximum after 2 hr, and was substained in the nucleus after 7 hr (Fig. 2B), which was totally different from kinetics of NF-kB activation by death factor TNF that nuclear complex disappeared in 1 hr (14). We also tested another compound SN-38. SN38 is an active metabolite of CPT11 in vivo and responsible for CPT-11 cytotoxicity, and was reported to have very strong growth inhibitory in cell culture. As shown Fig.1C, it also induced nuclear translocation of NF-kB in dose-dependent manner. This prolonged activation of NF-κB was independent of new gene products because it cannot blocked by transcription inhibitor actinomycin D and protein synthesis inhibitor cycloheximide (unpublished data). In the HT1080I, which expressed a super-repressor form of the NF-kB inhibitor IkBa, either CPT or SN38 could not induced nuclear traslocation of NF-kB(Fig. 1, lane 5-8).

Enhancement of CPT killing by inhibition of NF-kB. To test the NF-kB's role in CPT killing, the HT1080V and HT1080I were treated with CPT and SN-38 for 24 hours. As shown in Fig. 2, in absence of activation of NF-kB, CPT and SN38 cytotoxicity were enhanced. HT1080I killed by CPT was about 60% compared with HT1080V 20% at 5 mM for 24 hr (Fig. 2A). HT1080I killed by SN38 was 80% compared with HT1080V 50% at 2 mg/ml for 24 hr (Fig. 2B). Because Bcl-2 family have been implicated in resistance of cancer cell of chemotheraputic compounds including CPT (17), to rule out the possibility of Bcl-2 and Bcl-x_l expressed or induced at different level in HT1080V and I cells, Western blot were performed to examine the expression level of Bcl-2 and Bcl-x_l. As shown in Fig. 2C and D, Bcl-2 and Bcl-x_l were expressed at the similar basal level in HT1080V and HT1080I cells, and the level remained unchanged after CPT and SN38 treatment 4 hr.

CPT-induced apoptosis was dependent of Caspases. Apoptosis (programed cell death) is characterized by a structural changes in nucleus and cytoplasm, including membrane blebbing, chromatin condensation and internucleosomal DNA fragmentation (6-9). The family of interleukin-1-converting enzyme-like protease (ICE) recently renamed caspases have been shown that it play a critical role in apoptosis induced by multiple stimuli such as TNF and chemotherapy agents(). Although it is well indicated that CPT induce cell death through apoptotic mechanism, it remains possible that enhance killing by blocking NF-kB is through different mechanism. TUNEL assay detected DNA double-strand break by labeling free 3'-OH DNA ends with terminal deoxynucleotidyl transferase. It preferentially labels apopotosis in comparison to necrosis; thereby discriminating apoptosis from necrosis and from primary DNA strand breaks induced by antitumor drugs. As shown in Fig. 3, both the HT1080V and I cell treated by SN38 were stained positively. In HT1080I cells there were more apoptotic bodies than in HT1080V cells (compared panel B with D). Recently, It was reported that CPT-induced apoptosis is independent of caspases in some cell type, however, they use very specific and tetripeptide inhibitor that might not inhibit some caspases. Z-VAD is a new broad spectrum and specific tripeptide inhibitor of the caspase family that have widely been used in literature recently (15, 16). In order to accurately determine

whether Topo I-induced apoptosis can be blocked by caspase inhibitor, the cell death ELISA that also is a diagnostic test for apoptosis was performed. As shown in Fig 4, Z-VAD effectively inhibited DNA and histon released from cell and blocked CPT-induced apoptosis in HT1080I cells.

Discussion

In this report, we found Topo I inhibitors CPT and SN38 strongly activated NF-kB in human fibrosarcoma cell line HT1080. However, mechanism of activation of NF-kB is not clear. First, because CPT and SN38 induced nuclear translocation which is blocked by super-repressor IkBa, it suggest that activation of NF-kB by those compounds is through a pathway activates a common and unclear target IkB kinase (12). It has been proposed that DNA strand break, which is produced during oxidative stress, can lead to activation of NF-kB (12). However, activation of NF-kB by CPT cannot be abolished by an antioxidant NAC but not by PDTC. It suggest that DNA strand break may trigger an oxidative stress-independent and PDTC-inhibitable signaling cascade leading to NF-κB activation (12). Second, TNF leads to the rapid and transient activation of NF-kB with peak 30 min and disappearing in 1 hr. Persistent activation of NF-kB by CPT was different from the kinetics induced by TNF. It suggest that CPT may be through some unique intracellular signaling pathway to phosphate and degrade both IkBa and IkBb( ). In future study, it will be a challenge to identify this pathway so that we are able to block this pathway and increase cell sensitivity to Topo I inhibitors.. There were a couple of report that CPT-induced apoptosis occurred without a detectable increase in ICE-like or cystein protease P32 (CPP32)-like protease activity, internucleosomal DNA fragmentation was suppressed by serine protease inhibitors and the trypisin-like protease appeared to play a significant role in the CPT-induced apoptosis of HL-60 cell and Hep 3B (19, 20). By using a broader spectrum of caspase inhibitor, our result demonstrates that enhancement of SN38-induced apoptosis is dependent on caspases. These results may reflect a variation of cell type.

There are several mechanisms reported to effect the cell sensitivity to CPT-induced apoptosis (2-4). For example, overexpression of P-glycoprotein in lung and colon cancer was found to reduce the accumulation and cytotoxicity of topotecan. The cytotoxicity of CPT-11 is also reduced in P-glycoprotein expressing cells (). Bcl-2 families have been implicated as key regulators of DNA damage-mediated apoptosis. Overexpression of Bcl-2 inhibited CPT cytotoxity in murine IL-3 dependent prolymphoid progenitor cell line. In our cell model system, Bcl-2 and Bcl-x_l were constitutively expressed in the HT1080V and I cells, it seems not responsible for sensitivity to CPT and SN38. The other resistance mechanism is involved in the altered drug-target interaction. There are a couple of reports that some cells are resistant to CPT because of topo-1 mutation. Recent studies have demonstrated that the continuous exposure to CPT in cell culture and tumour-bearing animals lead to development of cell clones resistant to CPT derivatives. In those cases, either topo level is reduced compared to sensitive cell, or the enzyme is mutated and drug-insensitive (21-26). In inhibition of NF-kB activation, cell killing by CPT was enhanced. In future, it will also be very interesting to test whether CPT-resistant cell can be reversed to sensitive cell by inhibition of NF-kB. Overexpression of mutant E2F1 transcription factor in fibroblasts lengthened S phase increased cell sensitivity to CPT (27). On the contrary to E2F1, we found the trancription factor NF-kB that reduced the CPT killing power. At this time point, we are not clear whether protection mechanism of NF-kB is related to the cell cycle. NF-kB is universally preexisted and can be induced by various stimuli in most types of cells. In fact, CPT and SN38 also activated NF-kB in other cell types such as colorectal cancer cell line LOVO (unpublished data). Considering that NF-kB may be constitutively activated in some tumor cells such as breast cancer, it is very intriguing to combine gene therapy approach to specificly block activation of NF-kB pathway and enhance the CPT cytotoxicity. Since NF-kB is a transcription factor and is activated by CPT and its derivatives, we hypothesis that NF-kB complex moves to nucleus and activates κB-dependent gene(s) to inhibit the CPT-induced apoptosis. Since potentiation of CPT-induced apoptosis is dependent on caspases, it indicate that NF-κB-induced genes may directly or indirectly inhibit caspases. The experiment is in progress to try to

identify those down-stream genes that provide the survival function. The results may help to design more specific drugs or methods to reduce cell resistance and improve the efficacy of chemotherapy of topo I inhibitor CPTs.

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Figure legends

Figure 1. Activation of NF-kB in human fibrosarcoma cell line by CPT and SN38. A. and C. HT1080V and I cells were treated with indicated concentration of CPT and SN38 for 2hr. Nuclear translocation of NF-kB was detected by EMSA. A. Cell treated by CPT. B. Kinetics of activation of NF-kB by CPT. C. Cell treated by SN38

Figure 2. The inhibition of NF-kB activation enhanced CPT and SN38 cytotoxicity. Cells were treated by CPT or SN38 for 24-hr. Cell cytotoxicity were measured by crystal violet assay. The results represent the mean + SD of three experiments performed in duplicate or triplicate. A. Cell treated by CPT. B. Cell treated by SN38. C. Expression of Bcl-2 and Bcl-x_l. Cells were treated with 1uM CPT for indicated time. The protein level was determined by Western blot.

Figure 3. CPT killed cell through apoptotic mechanism as detected by TUNEL assay. Cell were treated or untreated with SN38 for 8 hr. The apoptotic cells were stained by dark brown colors.

Figure 4. Topo I inhibitor-induced apoptosis was inhibited by caspase inhibitor. Cells were preincubated with caspase inhibitor Z-VAD for 30 min, and then were treated by TNF 10 ng/ml, CPT and SN38, respectively. The results represent the mean $\pm$ SD of three independent experiments performed in duplicate or triplicate.